(12) EUROPEAN PATENT APPLICATION

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The title of the invention has been amended (Guidelines for Examination in the EPO, A-III, 7.3).

(54) Hybrid plasminogen activators.

(57) A hybrid plasminogen activator which comprises the five kringle domains of plasminogen linked to the B-chain of t-PA or u-PA via an amino acid sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA, or a derivative of a plasminogen activator comprising the serine protease domain of t-PA or u-PA, in which the catalytic site essential for plasminogen activator activity is blocked by a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electon-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C.

EP 0 297 882 A2

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Nov l Compounds

The present invention relates to a hybrid fibrinolytic enzyme, its preparation, pharmaceutical compositions containing it and its use in the treatment of thrombotic disease, and to enzyme derivatives for use in the treatment of thromboembolic diseases, in particular acute myocardial infarction.

European Patent No 0,009,879 discloses derivatives of in vivo fibrinolytic enzymes which are useful therapeutic agents for treating venous thrombosis. The derivatives are characterised by the active catalytic site on the enzymes being blocked by a group which is removable by hydrolysis such that the pseudo first order rate constant for hydrolysis is in the range 10^{-6} to 10^{-3} sec^{-1}.

EP-0155387 discloses fibrinolytically active hybrid protein which comprises one chain of a 2-chain protease linked to a chain of a different 2-chain protease, or to the same chain of the same protease, at least one of the chains in the hybrid protein being derived from a fibrinolytically active protease, such that the hybrid protein has a catalytic site essential for fibrinolytic activity which is optionally blocked by a removable blocking group.

Examples of the hybrid protein include plasmin A-chain linked to tissue-type plasminogen activator (t-PA) B-chain or urokinase B-chain.

The hybrid proteins may be prepared by mixing a chain of one protease with a chain of another protease, optionally with dialysis, under oxidative conditions.

Alternatively, the hybrid proteins may be prepared by taking the genetic information (DNA sequence) of each protein, cutting and ligating this to construct a new DNA sequence coding for the hybrid protein, and expressing this DNA in prokaryote or eukaryote hosts.

The sequence of amino acids making up the enzyme tissue-type plasminogen activator (t-PA) and the nucleotide sequence for the cDNA which codes for t-PA are known (see Pennica et al., 1983; Nature, 301, 214). t-PA is known to have fibrinolytic activity.

As used herein, the term tissue-type plasminogen activator (t-PA) denotes a plasminogen activator of the group having the immunological properties defined for t-PA at the XXVIII Meeting of the International Committee on Thrombosis and Haemostasis, Bergamo, Italy, 27 July 1982.

The amino acid sequence of various forms of t-PA are known. The abovementioned Nature 1983 reference discloses the sequence for the L-chain and the mature S-chain forms of t-PA, also discussed by Vehar et al., Biotechnology, 1984, 2, 1051-7 in which the processing of initially formed t-PA by removal of a pro-sequence to give the S-chain form is reported. Pohl et al., FEBS letters, 1984, Vol. 168 No.1, 29-32, refers to the N-terminal multiplicity of t-PA and discloses the U-chain form. The numbering system for the amino acid sequence of t-PA used herein is that described in the Nature 1983 reference for mature (S-chain) t-PA in which the N-terminal serine is numbered 1. By this system, L-chain t-PA has an N-terminal glycine residue at position -3 and U-chain t-PA has an N-terminal valine at position 4. References to t-PA herein are understood to include all such variant forms.

Native t-PA is composed of a B or light (L) and an A or heavy (H) chain. The B-chain contains the active site of the enzyme. The cleavage site for the conversion of t-PA from the single to the two-chain form is located between residues arg-275 and ile-276. In the two-chain form the chains are held together by a disulphide bridge formed between residues cys-264 in the A-chain and cys-395 in the B-chain.

It has been shown (Ny, T. et al, 1984; Proc. Natl. Acad. Sci. U.S.A., 81, 5355) that the A chain exhibits a number of structural and functional domains which are homologous to structures found in other plasma proteins: two triple disulphide-bonded structures or kringles, a growth-factor-like domain and a fibronectin-finger-like domain.

The region from amino acid residues 44 to 91 has been termed the "growth factor domain" (Banyai, L., et al FEBS Lett. 163, 37, 1983). The genetic information which codes for the major part of this domain, residues 51 to 86 inclusive, and partially codes for residues 50 and 87, lies on a single exon. The region from the first to last cysteine residues within this region, residues 51 to 84 inclusive, defines a triple-disulphide linked structure which will be referred to herein as the t-PA growth factor domain. Fibronectin has twelve finger-domains per monomer, responsible for fibrin-affinity (Eur. J. Biochem. 154, 15-29 (1986)). The amino acid sequences of these finger domains are known (EMBO J.4, 1755 - 1759 (1985); Eur. J. Biochem. 128, 605-623 (1982); Proc. Natl. Acad. Sci. USA 80, 137-141 (1983)). It has been shown (J. Biol. Chem. 260, 5328-5341 and 13666-13676 (1985)) that part of Factor XII shows structural homology with the finger-domains of fibronectin. It has also been shown (Banyai, L. et al, 1983; FEBS Lett., 163, 37)that a part of the t-PA enzyme shows structural homology with the finger-domains of fibronectin. This region from amino acid residue 6 to 43 has been termed the t-PA finger domain. The genetic information for this domain lies on a single exon (Ny, T. et al, 1984; Proc. Natl. Acad. Sci. U.S.A., 81, 5355). The term "finger domain" will be used hereinafter to refer to an amino acid sequence showing structural homology with the finger-domains of fibronectin, or the sequence of a fibronectin finger-domain itself.

The sequence of amino acids making up the enzyme urokinase-type plasminogen activator (u-PA) in its single chain and two chain forms (Verstraete, M. and Collen, D., 1986; Blood, 67, 1529) and the nucleotide sequence for the cDNA which codes for human u-PA (Holmes, W. E. et al, 1985; Bio/technology 3, 923-929) are known. Urokinase-type plasminogen activator is known to have fibrinolytic activity. The two chains of u-PA are termed the A- and B-chain. The B-chain contains the active site of the enzyme. The cleavage site for the

conversion of u-PA from the single to the two chain form is located between residues lys-158 and ile-159. In the two chain form the chains are held together by a disulphide bridge formed between residues cys-148 in the A-chain and cys-279 in the B-chain.

As used herein, the term urokinase-type plasminogen activator (u-PA) denotes a plasminogen activator of the group having the immunological properties defined for u-PA at the XXVIII Meeting of the International Committee on Thrombosis and Haemostasis, Bergamo, Italy, 27 July 1982.

The numbering system for the amino acid and nucleotide sequence of u-PA used her_in is that described in Holmes, W. E. et al, 1985 (op. cit.) in which the N-terminal serine residue is numbered 1.

In addition to the native forms of t-PA and u-PA described above, various muteins are also known, see for example EP-A-0201153, EP-A-0233013, EP-A-0199574, WO 86/01538, EP-A-0227462, EP-A-0253582, WO 86/04351, EP-A-0236040, EP-A-0200451, EP-A-0238304, EP-0225286, DE 3537176, WO 87/04722 and EP-A-0236289. References herein to t-PA and u-PA species include both native forms and muteins.

Plasmin is a two-chain serine protease which may be obtained by the cleavage of the single chain precursor, plasminogen, at a specific internal peptide bond. The amino acid sequence of human plasminogen is known (Wiman and Walters (1975) Eur.J. Biochem. 50, 489-494 and 58, 539-547; Wiman (1977) Eur. J. Biochem. 76, 129-137; Sottrup-Jensen et al. (1978) Fibrinolysis and Thrombolysis Vol. 3, 191-209, Raven Press, New York; and Sottrup-Jensen et al. (1978) Atlas of Protein Sequence and Structure Vol. 5, Suppl. 3, p91, National Biomedical Research Foundation, Silver Spring, MD). A partial nucleotide sequence coding for amino acid residues 272-790 of human plasminogen has also been described (Malinowski, D.P. et al., 1984, Biochemistry, 23, 4243-4250). The cleavage site of human plasminogen is located between residues arg-560 and val-561 (according to the sequence numbering of Sottrup-Jensen et al. (1978) Atlas of Protein Sequence (op.cit.)). Two species of plasminogen have been identified (F.J. Castellino, Chemical Reviews Vol. 81 p431 (1981)): glu_1 which has an N-terminal glutamic acid residue at position 1 and lys_{77} which has an N-terminal lysine residue at position 77. Glu-plasminogen is also easily converted by limited plasmic digestion to other modified forms with N-terminal valine (val_{78}) or methionine (met_{68}) (C. Miyashita, E. Wenzel and M. Heiden, Haemostasis 18, supp.1 pp 7-13 (1988)). References to plasminogen herein are understood to include all these species.

Recently a complete nucleotide sequence has been described (Forsgren, M., et al., 1987, FEBS Letters 213, 254-260). The nucleotide sequence predicts the existence of an extra, previously unreported, isoleucine residue near the N-terminus of the A-chain. This finding has been independently confirmed (McLean, J.N., et al., 1987, Nature 330, 132-137). Accordingly all sequence numbering (amino acid and nucleotide) below follows Forsgren et al. (1987). In this numbering sequence the plasminogen cleavage site is located between residues arg-561 and val-562 and the N-terminal modified forms are termed met_{69}, lys_{78} and val_{79}.

Plasminogen has five kringle structures. The region from the first to the last cysteine residue of each kringle structure, residues 84 to 162, 166 to 243, 256 to 333, 358 to 435 and 462 to 541 inclusive will be referred to herein as the K_1^p, K_2^p, K_3^p, K_4^p and K_5^p domains respectively.

According to the present invention there is provided a hybrid plasminogen activator which comprises the five kringle domains of plasminogen linked to the B-chain of t-PA or u-PA via an amino acid sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA.

It will be understood that by the term 'B-chain' is meant at least that portion of the B-chain containing the functional active centre of t-PA or u-PA, and preferably comprises residues 276-527 or 159-411 respectively.

The linking sequence of amino acids may be introduced synthetically during the preparation of the hybrid plasminogen activator (PA) and/or derived from native sequences.
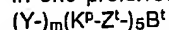
Native plasminogen includes cysteine residues at positions 548 and 558, C-terminal to plasminogen kringle 5, which participate in the interchain disulphide bridges of the two-chain plasmin form. In the preferred embodiment these residues are not present in the linking sequence.

It will be appreciated that to prevent cleavage of the plasminogen kringles from the t-PA or u-PA B-chain in vivo, the linking sequence should be chosen so as to avoid the presence of a site susceptible to trypsin-like proteolytic cleavage N-terminal to residue cys-264 of t-PA or cys-148 of u-PA, as appropriate.

Where the B-chain of t-PA is employed, the linking sequence of amino acids preferably comprises t-PA residues 264 to 275 inclusive, more preferably residues 262 to 275 inclusive.

Where the B-chain of u-PA is employed, the linking sequence of amino acids preferably comprises u-PA residues 148 to 158 inclusive, more preferably residues 137 to 158 inclusive.

In one preferred aspect, the hybrid PA may be represented symbolically as:

$(Y-)_m(K^p-Z^t-)_5B^t$

where B^t comprises residues 276-527 of t-PA, m is 0 or 1, preferably 1, each of the 5 values of K^p represents a kringle domain derived from plasminogen in sequence and Y and each of the 5 values of Z^t independently represents a bond or a linking sequence of amino acids which may be introduced synthetically during the preparation of the hybrid PA and/or derived from native plasminogen and/or t-PA sequences, the sequence Z_5^t comprising at least residues cys-264 and arg-275 of t-PA.

In a second preferred aspect, the hybrid PA may be represented symbolically as:
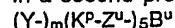
$(Y-)_m(K^p-Z^u-)_5B^u$

where B^u comprises residues 159-411 of u-PA, Y and each of the 5 values of Z^u independently represents a bond or a linking sequence of amino acids which may be introduced synthetically during the preparation of the

hybrid PA and/or derived from native plasminogen and/or u-PA sequences, the sequence Z_5^u comprising at least residues cys-148 and lys-158 of u-PA and m and each of the 5 values of K^p are as previously defined.

When m is 1, the sequence Y may take one of the values found in various forms of native plasminogen, such as the glu_1 or lys_{78} forms, that is plasminogen residues 1 to 83 or 78 to 83 r spectively.

Optionally, as taught generally in EP-A-0241210, the sequence Y may commence with one or more finger domains, defined herein as an amino acid sequence showing structural homology with the finger-domains of fibronectin, such as a fibronectin finger domain itself or a t-PA finger domain, preferably a t-PA finger domain.

Where a finger domain is derived from t-PA, the finger domain sequence may optionally extend at the N-terminus to include residues preceding residue 6 of native t-PA, such as residues 4 and 5, I to 5 or -3 to 5 respectively of the U-, S- or L-chain forms of native t-PA. The finger domain sequence preferably extends at the N-terminus to comprise residues 1 to 5 of native t-PA.

Each finger domain sequence may optionally be linked to a second sequence of amino acids which corresponds to the growth-factor domain of native t-PA. Thus, representing the finger domain sequence (for example residues 6 to 43 of t-PA) as F and the growth-factor domain sequence (residues 51 to 84 of t-PA) as G, Y comprises one or more N-terminal units of the form $A-F-X_1-$ and/or $A-F-X_2-G-X_3-$, where X_1, X_2 and X_3 represent bonds or linking sequences of amino acids which may be introduced synthetically during the preparation of the hybrid PA and/or be derived from native t-PA sequences adjacent the F and G domains and A is an optional N-terminal extension of the F domain.

In a preferred aspect, the linking sequence X_1 comprises amino acid residues selected from the residues 44 to 50 and 85 to 91, and more preferably comprises residues 44 to 50 and 88 to 91, of native t-PA, optionally linked at the C-terminus to a sequence of amino acids, such as -pro-gly-. The linking sequence X_2 preferably comprises residues selected from the residues 44 to 50 of native t-PA, and more preferably comprises residues 44 to 50. The linking sequence X_3 preferably comprises residues selected from the residues 85 to 91 of native t-PA and more preferably comprises residues 85 to 91, optionally linked at the C-terminus to a sequence of amino acids such as -pro-gly-.

It will be appreciated that to prevent cleavage of the additional domain(s) from one another or from the remainder of the hybrid plasminogen activator in vivo, inter-domain linking sequences and linking sequences between the additional domain(s) and the plasminogen activator molecule should be chosen so as to avoid the presence of a site susceptible to proteolytic cleavage. Thus, in particular, the linking sequence X_1 or X_3 will preferably end with a residue other than arginine or lysine.

Y preferably comprises up to 6 additional finger domains, more preferably up to 2, most preferably 1, each of which may optionally be linked to a growth factor domain.

Z_1, Z_2, Z_3 and Z_4 preferably represent the native plasminogen inter-domain sequences between plasminogen kringle domains 1 and 2, 2 and 3, 3 and 4 and 4 and 5, respectively.

Suitable sequences (Z_5^t) linking the C-terminal plasminogen kringle domain to the t-PA B-chain include:

1.   [AAPSTCGLRQYSQPQFR]

2.   [AAPSTCGLRQYSQPQFQ]

3.   [STCGLRQYSQPQFR]

(single letter amino acid notation) from which it can be seen that the sequences 1 and 2 consist of residues 542-544 of plasminogen and residues 263 to 275 of t-PA linked by a serine residue. The interposed serine residue can be identified with ser-545 of plasminogen or ser-262 of t-PA. In sequence 2, residue 275 of t-PA has been replaced by glutamine in accordance with EP-A-0233013. Sequence 3 consists of residues 262 to 275 of t-PA.

The preferred sequence (Z_5^u) linking the C-terminal plasminogen kringle domain to the u-PA B-chain is:
[AAPSFPSSPPEELKFQCGQKTLRPRFK]
(single letter amino acid notation) from which it can be seen that the sequence consists of residues 542-546 of plasminogen and residues 137 to 158 of u-PA.

The preferred hybrid PA's of the invention have the following structures:

1. Plg 1-541[AAPSTCGLRQYSQPQFR]B^t

2. Plg 1-541[AAPSTCGLRQYSQPQFQ]B^t

3. Plg 1-541   [STCGLRQYSQPQFR]B^t

where Plg 1-541 represents residues 1-541 of plasminogen (that is, including kringles 1 to 5), B^t is as previously defined and the symbols in brackets represent amino acid residues according to the single letter amino acid notation, including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof.

The hybrid PA of the invention may be derivatised to provide pharmaceutically useful conjugates analogous

to known PA-containing conjugates, for example:

(a) an enzyme-protein conjugate as disclosed in EP-A-O 155 388, in which the catalytic site on the enzyme which is responsible for fibrinolytic activity is blocked by a human protein attached thereto by way of a reversible linking group;

(b) an enzyme-protein conjugate as disclosed in EP-A-O 152 736, comprising at least one optionally blocked fibrinolytic enzyme linked by way of a site other than the catalytic site responsible for fibrinolytic activity to at least one human protein;

(c) a protein-polymer conjugate as disclosed in EP-A-0183503 comprising a pharmaceutically useful protein linked to at least one water soluble polymer by means of a reversible linking group; or

(d) an enzyme conjugate as disclosed in EP-A-0184363 comprising a plurality of fibrinolytic enzymes linked together through the active centres thereof by means of a removable blocking group.

The hybrid PA of the invention may take the place of a PA as the enzyme or (human) protein component, as appropriate, of any of the conjugates described above.

The above mentioned derivatives of the hybrid PA may be used in any of the methods and compositions described hereinafter for the hybrid PA itself.

In a further aspect, the invention provides a process for preparing hybrid plasminogen activator according to the invention which process comprises expressing DNA encoding said hybrid plasminogen activator in a recombinant host cell and recovering the hybrid plasminogen activator product.

The DNA polymer comprising a nucleotide sequence that encodes the hybrid PA also forms part of the invention.

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis et. al., Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982 and DNA Cloning vols I, II and III (D.M. Glover ed., IRL Press Ltd).

In particular, the process may comprise the steps of:

i) preparing a replicable expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes said hybrid plasminogen activator;

ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said hybrid plasminogen activator; and

iv) recovering said hybrid plasminogen activator.

The invention also provides a process for preparing the DNA polymer by the condensation of appropriate mono-, di- or oligomeric nucleotide units.

The preparation may be carried out chemically, enzymatically, or by a combination of the two methods, in vitro or in vivo as appropriate. Thus, the DNA polymer may be prepared by the enzymatic ligation of appropriate DNA fragments, by conventional methods such as those described by D. M. Roberts et al in Biochemistry 1985, 24, 5090-5098.

The DNA fragments may be obtained by digestion of DNA containing the required sequences of nucleotides with appropriate restriction enzymes, by chemical synthesis, by enzymatic polymerisation, or by a combination of these methods.

Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

Enzymatic polymerisation of DNA may be carried out in vitro using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less.

Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer at a temperature of 4°C to ambient, generally in a volume of 50µl or less.

The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J. Gait, H.W.D. Matthes M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B. S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams et al., Journal of the American Chemical Society, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes et al., EMBO Journal, 1984, 3, 801. Preferably an automated DNA synthesizer is employed.

The DNA polymer is preferably prepared by ligating two or more DNA molecules which together comprise a DNA sequence encoding the hybrid PA.

.The DNA molecules may be obtained by the digestion with suitable restriction enzymes of vectors carrying the required coding sequences.

The precise structure of the DNA molecules and the way in which they are obtained depends upon the structure of the desired hybrid PA product. The design of a suitable strategy for the construction of the DNA molecule coding for the hybrid PA is a routine matter for the skilled worker in the art.

In the preferred embodiment, the DNA molecule may conveniently be prepared by ligating a first DNA molecule encoding kringles 1 to 5 of plasminogen with one or more DNA molecules which, together with said

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first DNA molecule, encode the B-chain of t-PA or u-PA, such that the ligated molecul codes for the amino acid linking sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA.

By way of example and guidanc , two strategies for the construction of DNA molecules coding for two specific hybrid plasminogen activators are described below

Strategy A.

The first DNA molecule may be obtained by the digestion of a first vector carrying the required part of the plasminogen coding sequence. Conveniently the native plasminogen sequence is modified to introduce a new NarI site 3' to the K_5^P coding region, preferably at nucleotide position 1758, and any NarI site formerly present in the vector is destroyed. The first vector preferably also carries at least part of the t-PA coding sequence including the SstI site at nucleotide position 1417 (Pennica et al., 1983, op. cit.) in the B-chain coding region and the portion of the B-chain coding region 3' thereof. Digestion of the first vector with restriction enzymes NarI and SstI thus provides a DNA molecule coding for plasminogen kringles 1 to 5 and part of the t-PA B-chain, and carrying vector functions.

A second DNA molecule may be obtained by the digestion of a second vector carrying the t-PA B-chain coding sequence. Digestion of the second vector with suitable restriction enzyme(s) such as BanII provides a DNA molecule coding for part of the t-PA B-chain. The Ban II sticky end is compatible with the SstI sticky end of the first DNA molecule.

The first and second DNA molecules are linked via an oligonucleotide linker having suitable sticky ends compatible with the NarI and BanII sticky ends of said DNA molecules, and having a structure such that, upon ligation of the first and second DNA molecules with the linker, the resulting molecule codes for an amino acid linking sequence comprising the t-PA cleavage site between residues 275-276 and the cysteine residue 264 of t-PA. Conveniently, the oligonucleotide linker has the following structure:

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5'  CG.CCG TCG ACC TGC GGC CTG AGA CAG TAC AGC
3'      GGC AGC TGG ACG CCG GAC TCT GTC ATG TCG


    CAG CCT CAG TTT CGC ATC AAA GGA GGG CT 3'
    GTC GGA GTC AAA GCG TAG TTT CCT C        5'
```

The ligation of the first and second DNA molecules with the above linker may be carried out sequentially or, more preferably, in a single step. Ligation of the BanII and SstI sticky ends of the molecules reforms the t-PA B-chain coding region. The resulting sequence at the reconstructed NarI site is believed to be as follows:

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              NarI
5' ...GCG GCG CCG TCG ACC TGC GGC CTG... 3'
3' ...CGC CGC GGC AGC TGG ACG CCG GAC... 5'
     ala ala pro ser*thr cys gly leu
     542 543 544     263 264 265 266
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Amino acids marked 542-544 are the C-terminal residues of a peptide sequence comprising residues 1 to 544 of plasminogen. The serine residue marked * may be identified with serine 545 of plasminogen or serine 262 of t-PA. Amino acids marked 263-266 are the N-terminal residues of a peptide sequence comprising residues 263-527 of t-PA.

The said first vector carrying the required part of the plasminogen coding sequence, that is, kringles 1 to 5, and with a NarI site introduced at nucleotide position 1758, may conveniently be obtained by the digestion with restriction enzymes HindIII and AhaII and, separately, AhaII and DdeI of a vector carrying the plasminogen gene, in which any existing vector NarI site has been removed by conventional methods. The HindIII site is located in the 5' untranslated region, and the AhaII site at nucleotide position 1572 and the DdeI site at nucleotide position 1748 are in the kringle 5 coding region of the plasminogen gene. The HindIII-AhaII and

AhaII-DdeI fragments may be ligated together and to an oligonucleotide linker having a DdeI sticky end, coding for the remainder of the kringle 5 coding region and containing a NarI site. The linker is preferably also provided with a BglII sticky end, such that the resulting DNA molecule, coding for plasminogen kringles 1 to 5, has a HindIII and a BglII sticky end and may be held in a suitable vector such as pTRE12 (EP-A-0 201 153) between the HindIII and BglII sites thereof, creating vector (A).

In order to introduce the part of the t-PA B-chain coding sequence which is 3' to the SstI site into the vector which carries the plasminogen $K_1^P-K_5^P$ coding region, the BglII fragment encoding the mature t-PA polypeptide obtained from a suitable vector (B) coding therefor, such as pTRE15 (EP-A-0 201 153), is inserted in the BglII site of the vector (A). The unwanted portion of the resulting vector may then be excised by digestion with restriction enzymes NarI and SstI to yield the said first DNA molecule encoding plasminogen kringles 1 to 5, t-PA B-chain 3' to the SstI site and vector functions.

The said second vector carrying the required part of the t-PA coding sequence, that is, the B-chain, may be for example a vector (B) encoding the mature t-PA polypeptide, such as pTRE15 (EP-A-0 201 153).

Strategy B

The first DNA molecule may be obtained by the digestion of a first vector carrying the required part of the plasminogen coding sequence. Conveniently the native plasminogen sequence is modified to introduce a new NarI site 3' to the K_5^P coding region, preferably at nucleotide position 1758, and any NarI site formerly present in the vector is destroyed. The first vector conveniently also has a BglII site positioned such that digestion of the first vector with restriction enzymes NarI and BglII provides a DNA molecule coding for plasminogen kringles 1 to 5 and carrying vector functions.

A second DNA molecule may be obtained by the digestion of a second vector carrying the u-PA B-chain coding sequence. Conveniently the native u-PA sequence is first modified by the introduction of an SstI site 5' to the u-PA B-chain coding region. The second vector preferably also carries at least part of the u-PA A-chain coding sequence 5' to the B-chain coding sequence and conveniently has a BglII site 3' to the B-chain coding sequence. Digestion of the second vector with restriction enzymes BglII and SstI thus provides a DNA molecule coding for u-PA B-chain and part of the A-chain.

The first and second DNA molecules are linked via an oligonucleotide linker having suitable sticky ends, preferably a 5' NarI sticky end and a 3' SstI sticky end, and having a structure such that, upon ligation of the first and second DNA molecules with the linker, the resulting molecule codes for an amino acid linking sequence comprising the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA. Conveniently, the oligonucleotide linker has the following structure:

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5'  CG CCT TCA TTT CCC TCC TCT CCT CCA GAA GAG CT 3'
3'     GGA AGT AAA GGG AGG AGA GGA GGT CTT C     5'
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The ligation of the first and second DNA molecules with the above linker may be carried out sequentially or, more preferably, in a single step. The resulting sequence at the reconstructed NarI and SstI sites is believed to be as follows:

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```
                      NarI
                   ⌢‾‾‾⌒‾‾‾⌢
                        *
 5   5' ... TGT GCG GCG CCT TCA TTT CCC TCC TCT CCT CCA GAA
     3' ... ACA CGC CGC GGA AGT AAA GGG AGG AGA GGA GGT CTT
            cys ala ala pro ser phe pro ser ser pro pro glu
10          541 542 543 544 545 546 137 138 139 140 141 142


                   SstI
                ⌢‾‾‾⌒‾‾‾⌢
15               *   *   *
            GAG CTC AAA ... 3'
            CTC GAG TTT ... 5'
            glu leu lys
20          143 144 145
```

25   Amino acids marked 541-546 are the C-terminal residues of a peptide sequence comprising residues 1 to 546 of plasminogen. Amino acids marked 137-145 are the N-terminal residues of a peptide sequence comprising residues 137-411 of u-PA.

The nucleotides marked * have been substituted for the native nucleotides in the course of creating the NarI and SstI restriction sites, without altering the encoded amino acids.

30   The said first vector carrying the required part of the plasminogen coding sequence, that is, kringles 1 to 5, and with a NarI site introduced at nucleotide position 1758, may conveniently be obtained as described in strategy A above.

The said second vector carrying the required part of the u-PA coding sequence, that is, the B-chain, and with an SstI site 5' to the B-chain coding region, may conveniently be obtained by conventional mutagenesis

35   techniques to introduce the SstI site into a vector (c) encoding the u-PA polypeptide, conveniently at nucleotide position 563. The u-PA polypeptide is conveniently held between the HindIII and BglII sites of a suitable vector, such as pTRE12 (EP-A-0 201 153).

The expression of the DNA polymer encoding the hybrid PA in a recombinant host cell may be carried out by means of a replicable expression vector capable, in the host cell, of expressing the DNA polymer. The

40   expression vector is novel and also forms part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment, encode the hybrid PA, under ligating conditions.

45   The ligation of the linear segment and more than one DNA molecule may be carried out simultaneously or sequentially as desired.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic, such as E. Coli, or eukaryotic, such as mouse C127, mouse myeloma, chinese hamster ovary or yeast. Suitable vectors include

50   plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis et al., cited above. Polymerisation and ligation may be performed as described above for the preparation of the DNA polymer. Digestion with restriction enzymes may be performed in an appropriate buffer

55   at a temperature of 20°-70°C, generally in a volume of 50μl or less with 0.1-10μg DNA.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al., cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

60   The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as E. coli may be treated with a solution of $CaCl_2$ (Cohen et al, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells.

65   The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing th transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis et al and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The hybrid PA expression product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as E. coli it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium.

The DNA polymer may be assembled into vectors designed for isolation of stable transformed mammalian cell lines expressing the hybrid PA; e.g. bovine papillomavirus vectors or amplified vectors in chinese hamster ovary cells (DNA cloning Vol.II D.M. Glover ed. IRL Press 1985; Kaufman, R.J. et al., Molecular and Cellular Biology 5, 1750-1759, 1985; Pavlakis G.N. and Hamer, D.H., Proceedings of the National Academy of Sciences (USA) 80, 397-401, 1983; Goeddel, D.V. et al., European Patent Application No. 0093619, 1983).

It will be appreciated that, depending upon the host cell, the hybrid PA prepared in accordance with the invention may be glycosylated to varying degrees. Furthermore, as observed by Pohl et.al., Biochemistry, 1984, 23, 3701-3707, varying degress of glycosylation may also be found in unmodified, naturally occurring t-PA. Plasminogen also exhibits varying degrees of glycosylation (Hayes M.L, J. Biol. Chem. 254: 8768, 1979). Mutant forms of the hybrid PA are also contemplated in which glycosylation sites are removed by genetic engineering techniques, for example as taught in EP-A-0238304, EP-A-0225286, DE-3537176, WO 87/04722 or EP-0236289. The hybrid PA of the invention is understood to include such glycosylated variations.

It will also be appreciated that, depending upon expression conditions, the hybrid PA prepared in accordance with the invention may exist in the single or two chain forms or mixtures thereof. The invention extends to all such forms.

The hybrid PA of the invention comprises the B-chain of native t-PA or u-PA linked to an A-chain comprising the five kringle domains derived from plasminogen and linking sequence of amino acids comprising residues 264 and 275 of t-PA or residues 158 and 148 of u-PA.

This hybrid PA A-chain may be employed as one chain of a fibrinolytically active hybrid protein such as disclosed in EP-0 155 387. The hybrid A-chain, DNA encoding the hybrid A-chain and a hybrid protein comprising the hybrid A-chain linked to the B-chain of a fibrinolytically active protease, the catalytic site of which is optionally blocked by a removable blocking group, all form part of the invention.

The hybrid A-chain may be prepared by separation from the B-chain thereof by mild reduction. Alternatively the hybrid A-chain may be prepared by expressing DNA coding therefor in a recombinant host cell and recovering the hybrid A-chain product. The hybrid protein comprising the hybrid A-chain linked to the B-chain of a fibrinolytically active protease may be prepared by (a) mixing said A- and B-chains under oxidative conditions; or (b) ligating DNA encoding said A-chain to DNA encoding said B-chain and expressing the ligated DNA in a prokaryote or eukaryote host; and thereafter optionally blocking the catalytic site of the hybrid protein with a removable blocking group. The oxidation and reduction conditions are as generally described in EP-A-0 155 387.

The resulting hybrid protein may be used in any of the methods and compositions described hereinafter for the hybrid PA itself.

The hybrid PA of the invention or conjugate thereof can be further derivatised such that any catalytic site essential for fibrinolytic activity is optionally blocked by a removable blocking group.

As used herein the expression 'removable blocking group' includes groups which are removable by hydrolysis at a rate such that the pseudo-first order rate constant for hydrolysis is in the range of 10^{-6} sec^{-1} to 10^{-2} sec^{-1}, more preferably 10^{-6} sec^{-1} to 10^{-3} sec^{-1}, in isotonic aqueous media at pH 7.4 at 37°C.

Such blocking groups are described in European Patent No.0009879 and include acyl groups such as optionally substituted benzoyl or optionally substituted acryloyl.

Suitable optional substituents for benzoyl blocking groups include halogen, C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkanoyloxy, C_{1-6} alkanoylamino, amino or p-guanidino.

Suitable optional substituents for acryloyl blocking groups include C_{1-6} alkyl, furyl, phenyl or C_{1-6} alkylphenyl.

In a preferred aspect, the removable blocking group is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electon-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C.

Preferably the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 2.75×10^{-1} s^{-1}, preferably 6.0×10^{-5} to 2.5×10^{-4} s^{-1}, more preferably 6.0×10^{-5} to 2.0×10^{-4} s^{-1}, still more preferably 6.0×10^{-5} to 1.5×10^{-4} s^{-1} and most preferably 7.0×10^{-5} to 1.5×10^{-4} s^{-1}.

Preferably, the 2-aminobenzoyl group is substituted with a halogen atom in the 4-position.

Preferably, the halogen atom is fluorine, chlorine or bromine.

When the group is further substituted, preferred groups include C_{1-6} alkyl, C_{1-6} alkoxy and C_{1-6} alkenyl substituents in the 3- or 5-positions of the ring.

Preferably, the blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

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The abovementioned substituted 2-aminobenzoyl blocking groups may be used to derivatise any plasminogen activator comprising the serin protease domain of t-PA or u-PA. This novel group of block d enzymes within the broad scope of EP-0009879 but not specifically disclosed therein have been found to undergo reg n ration of functional active centr s at rates corresponding to pseudo first-order rate constants

5 of 6×10^{-5} to 4×10^{-4} sec^{-1}. The latter range of rate constants is especially appropriate for the provision of fibrinolytic activity during the treatment by thrombolytic agents of acute myocardial infarction.

Accordingly, in a further aspect, the invention provides a derivative of a plasminogen activator comprising the serine protease domain of t-PA or u-PA, in which the catalytic site essential for plasminogen activator activity is blocked by a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and

10 optionally further substituted with one or more weakly electron-withdrawing or electon-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C.

15 A suitable detergent for use in the buffer is the product having the tradename Tween 80.

Suitable examples of the blocking group are as described above.

The term plasminogen activator includes human tissue plasminogen activator (t-PA) and urokinase (both high and low molecular weight forms). Such enzymes are obtained from mammalian blood, urine or tissues or by recombinant DNA methods where heterologous host organisms such as bacteria, yeasts, fungi or

20 mammalian cells express genes specifying the enzymes. The term also includes:

(a) fibrinolytically active hybrid proteins disclosed in EP-A-0155387, such as lys$_{78}$ plasmin A-chain/ile$_{276}$ t-PA;

(b) protein conjugates disclosed in EP-A-0152736, such as urokinase linked to reversibly blocked plasmin;

25 (c) derivatives of fibrinolytic enzymes disclosed in EP-A-0155388 in which the human protein component comprises the serine protease domain of t-PA or urokinase, such as urokinase reversibly linked to the active centre of human plasmin;

(d) conjugates comprising a fibrinolytic enzyme linked to a water-soluble polymer by means of a reversible linking group as disclosed in EP-A-0183503; and

30 (e) genetically engineered derivatives including muteins of naturally occurring plasminogen activators such as those disclosed in EP-A-0201153, EP-A-0207589, WO-8604351, EP-0041766, EP-0213794, EP-0199574, EP-A-0240334, EP-A-0241208, EP-A-0241209, EP-A-0241210, EP-A-0233013, European Patent Application No. 88301897.0 and USSN 163959, European Patent Application No. 88304626.0, EP-A-0213794, EP-A-0231883, WO 8704722, EP-A-0242836, EP-A-0234051, EP-A-0253582, EP-

35 A-0253241, WO-8604351, EP-A-0236040, EP-A-0200451, EP-A-0238304, EP-A-0225286, DE-3537176, EP-A-0236289, WO-8601538, EP-0227462, AU-8661804, WO-8703906 and EP-0199574, such as des(cys$_{51}$-asp$_{87}$)t-PA; provided that the enzymes listed in (a) to (e) comprise the serine protease domain of t-PA or urokinase.

Preferred derivatives of this invention include the following:

40 4-fluoro-2-aminobenzoyl urokinase,

4-chloro-2-aminobenzoyl urokinase or t-PA, and

4-bromo-2-aminobenzoyl t-PA;

2-amino-4-chlorobenzoyl(lys$_{78}$ plasmin A-chain)ile$_{276}$ t-PA;

the 4-chloro- and 4-fluoro-2-aminobenzoyl derivatives of the conjugates in which urokinase (high or low

45 molecular weight) is linked reversibly to the active centre of human plasmin;

the 4-chloro-2-aminobenzoyl derivative of des(cys$_{51}$-asp$_{87}$)t-PA; and, most preferably,

4-chloro-2-aminobenzoyl plg 1-544/t-PA 262-527 (two chain) including the glu$_1$ and lys$_{78}$ variants and mixtures thereof, and

4-chloro-2-aminobenzoyl plg 1-541/t-PA 262-527 (two chain) including the glu$_1$ and lys$_{78}$ variants and mixtures

50 thereof.

The present invention also provides a process for preparing a derivative of the invention, which process comprises reacting the plasminogen activator with a blocking agent JK in which J is a locating group which mediates binding of the agent to the catalytic site of the enzyme and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly

55 electron-withdrawing or electron-donating groups.

Examples of group J include 4-amidinophenyl and 2-chloro-4-amidinophenyl.

Preferred agents JK are 4-amidinophenyl 2'-amino-4'-fluorobenzoate; 4-amidinophenyl 2'-amino-4'-chlorobenzoate and 4-amidinophenyl 2'-amino-4'-bromobenzoate.

Blocking agents JK as above defined are novel and form part of the invention.

60 The blocking reactions are preferably carried out in aqueous media, at a pH in the range 7.0 to 8.5 and at temperatures in the range 0°C to 30°C.

The preferred concentration range for the blocking agent is 0.01 to 2.0 millimolar, and the preferred enzyme concentration range is 1 to 500 micromolar.

In certain cases, particularly with plasminogen activators such as t-PA and those structurally related to or

65 derived from plasminogen, it is desirable to carry out the blocking reaction in the presence of enzyme

solubilising or stabilising additives such as 1-arginine, 1-lysine or 6-aminohexanoic acid. The materials have been found to be compatible with the blocking agents of the invention.

The blocking agents JK may be prepared by the reaction of the appropriate substituted benzoic acid with th appropriate alcohol in an organic base such as pyridine and in the presence of a condensing agent such as dicyclohexylcarbodiimide. Th condensation reaction may be performed without prior protection of the 2-amino substituent in the acid component.

· The precursor substituted anthranilic acids may be prepared by a variety of methods known in the art. In general, the relative positions of the desired substitutents will determine the chosen starting material. Where an optional substituent such as methyl or methoxy is required, this is generally present in the starting material. The halo, amino and carboxylic acid groups are successively introduced, as necessary, in any appropriate order, by conventional aromatic substitution. Amino and carboxylic acid groups may be provided in precursor form. Amino groups may be obtained from nitro groups by reduction. Carboxylic acid groups may be obtained from methyl groups by oxidation or from ester or thioester groups by hydrolysis. During successive introduction of substituents, it may be necessary to protect amino and/or carboxylic acid groups.

One preferred route comprises the preparation of an appropriate aminotoluene derivative by reduction of the corresponding nitrotoluene, protection of the amino group (for example by acetylation), oxidation of the methyl function to a carboxylic acid group (for example with potassium permanganate) and removal of the protecting group by hydrolysis.

4-Halo-5-methyl anthranilic acids may be prepared by firstly protecting the carboxylate in 4-amino-m-toluic acid with thionyl chloride dimethylamine (to give the dimethylamide), then nitrating the ring at the 2-position with excess butyl lithium/acetyl nitrate, diazotising the free amino group and substituting a halogen atom using the Sandmeyer reaction, reducing the nitro group to give the amine in the 2-position and finally hydrolysing the dimethylamide to the free acid. The same compounds (or, for example, the corresponding 5-methoxy derivatives) may also be prepared from 4-nitro toluene or 4-nitro anisole by halogenation at the 2-position with chlorine/aluminium chloride, reduction to the amine which is then protected by acetylation, insertion of a carboxyl precursor by electrophilic substitution with aluminium chloride and chloroformate ester or thioester and finally hydrolysis of both acetyl and ester/thioester groups to liberate the unprotected amine and carboxyl functions.

The hybrid PA and derivatives of the invention are suitably administered in the form of a pharmaceutical composition.

Accordingly the present invention also provides a pharmaceutical composition comprising a hybrid PA or derivative of the invention in combination with a pharmaceutically acceptable carrier.

The compositions according to the invention may be formulated in accordance with routine procedures as pharmaceutical compositions adapted for intravenous administration to human beings.

Typically compositions for intravenous administration are solutions of the sterile enzyme in sterile isotonic aqueous buffer. Where necessary the composition may also include a solubilising agent to keep the hybrid PA or derivative in solution and a local anaesthetic such as lignocaine to ease pain· at the site of injection. Generally, the hybrid PA or derivative will be supplied in unit dosage form for example as a dry powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of protein in activity units. Where composition comprises a derivative of the invention or where the hybrid PA includes a removable blocking group, an indication of the time within which the free protein will be liberated may be given. Where the protein is to be administered by infusion, it will be dispensed with an infusion bottle containing sterile pharmaceutical grade 'Water for Injection' or saline. Where the protein is to be administered by injection, it is dispensed with an ampoule of sterile water for injection or saline. The injectable or infusable composition will be made up by mixing the ingredients prior to administration.

The quantity of material administered will depend upon the amount of fibrinolysis required and the speed with which it is required, the seriousness of the thromboembolic condition and position and size of the clot. The precise dose to be employed and mode of administration must <u>per force</u> in view of the nature of the complaint be decided according to the circumstances by the physician supervising treatment. However, in general, a patient being treated for a thrombus will generally receive a daily dose of from 0.01 to 10 mg/kg of body weight, such as 0.10 to 2.0mg/kg, either by injection in for example up to five doses or by infusion.

Within the above indicated dosage range, no adverse toxicological effects are indicated with the compounds of the invention.

Accordingly, in a further aspect of the invention there is provided a method of treating thrombotic diseases, which comprises administering to the sufferer an effective non-toxic amount of hybrid PA of the invention.

In another aspect the invention provides the use of a hybrid PA of the invention for the manufacture of a medicament for the treatment of thrombotic diseases.

The invention also provides a hybrid PA of the invention for use as an active therapeutic substance and in particular for use in the treatment of thrombotic diseases.

The present invention further provides a method of treatment of thromboembolic diseases, in particular acute myocardial infarction, which method comprises administering to the sufferer an effective, non-toxic amount of a derivative of the invention.

The invention also provides the use of a derivative of the invention for the manufacture of a medicament for the treatment of thromboembolic diseases, in particular acute myocardial infarction.

In a further aspect, the invention provides a derivative of the invention for use as an active therapeutic

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substance, in particular for the treatment of thromboembolic diseases such as acute myocardial infarction. The following Methods and Examples illustrate the invention.

I. General Methods used in Examples

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(i) DNA cleavage

In general the cleavage of about 1µg of plasmid DNA or DNA fragments was effected using about 5 units of a
10 restriction enzyme (or enzymes) in about 20µl of an appropriate buffer solution.

(ii) Generation of blunt ends:

If blunt ends were required they were produced by treating the DNA preparation with DNA Polymerase I,
15 Klenow fragment as described by Maniatis et al, (Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, 1982), or alternatively (where indicated) by digestion using Mung Bean nuclease (Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

(iii) Generation of 'Sticky ends' using linkers:
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Short kinased oligonucleotide linkers encoding single or multiple restriction sites were ligated onto blunt ended fragments, the linker(s) was digested with the appropriate restriction endonuclease producing the required 'sticky end' necessary for further manipulation. (Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, 1982)

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(iv) Ligation of DNA Fragments:

Ligation reactions were carried out as described in Maniatis et al, (Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).
30   (v) Transformation of plasmid DNA into E.coli HB101 or E.coli DH5α cells used competent HB101 or DH5α cells supplied by Gibco BRL (Paisley, Scotland), according to the manufacturers instructions.

(vi) Plasmid preparation:

35   Large scale preparation of plasmid DNA and plasmid mini-preparations were carried out as described in Maniatis et al, (Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, (1982)).

(vii) Isolation of DNA fragments from low-melting-point (LMP) agarose gels:

40   DNA fragments were isolated from LMP agarose gels as described by Maniatis et al, (Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). Alternatively the excised gel band was purified using GENECLEAN®, (Stratech Scientific, London) used according to the manufacturers instructions.

(viii) Oligonucleotides:
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Oligonucleotides were made on Applied Biosystems 381A DNA Synthesizer according to the manufacturers instructions and were kinased as described in Maniatis et al, op. cit. When used in plasmid construction the oligonucleotides were annealed by heating together at 95°C for 5 minutes and cooling slowly to room temperature. The annealed oligonucleotides were then ready for ligation. If four oligonucleotides were to be
50   annealed the annealing reaction was followed as described above but was carried out as 2 reactions each containing a pair of complementary oligonucleotides. After cooling the 2 reactions were mixed prior to ligation.

(ix) DNA sequencing

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(a) Single-strand method

Nucleotide sequence determination was carried out by the Sanger dideoxy chain termination method as described in Sanger. S., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA $\underline{74}$, 5463-5467 using
60   ^{35}S-labelled dATP.

(b) Double-strand method

Sequencing was carried out using 'Sequenase® (United States Biochemical Corporation) essentially
65   according to the manufacturers instructions.

(x) Transient expression of plasminogen activators from HeLa cells

(a) Small-scale

Cell preparation: cells were trypsinised and plated out at approx. 2.4×10^5 cells per 35mm dish and incubated in 1.5ml growth medium (this is Hepes buffered RPM1 1640 medium (041-2400) containing 10% Serum (021-6010), 1% stock solution of penicillin/streptomycin (043-5070), 2% sodium bicarbonate solution (043-5080), 1% stock Glutamine (043-5030); Gibco, Paisley, Scotland) at 37°C in a humidified incubator in an atmosphere of 5% CO_2/95% air. After 72h the cells were refed, and used for transfection 24h later.

Transfection procedure: Cultures were changed to Eagles MEM (041-1095), 10% serum, 1% penicillin/streptomycin, 1% Glutamine and 1% non-essential amino acids (043-1140) 3h before transfection. The transfections used calcium coprecipitation as described in 'DNA Cloning' Ed. D.M. Glover (Chap. 15, C. Gorman). Glycerol shock and 5mM butyrate treatments were used. Plasminogen activator(s) secreted by transfected cells was harvested in 1.0ml RPMI 1640 medium (as above but serum-free, containing 4% soybean peptone (Sigma) for 24h and activity was measured using fibrin plates with reference to normal t-PA (J.H. Robinson, Thromb. Res., 1984; 33, 155).

Alternatively the HeLa cells were plated at 5×10^5 per dish, in which case the cells were refed after only 24h and then used as above.

(b) Large-scale

Cell preparation: cells were trypsinised and plated out at a density of approx. 2.5×10^6 cells per 175cm^2 flask in 30ml growth medium (above). After 72h an extra 25ml of growth medium was added and the cells were used for DNA transfection 24h later (as above). 25ml of harvest medium were used per flask.

Alternatively the cells were plated at a density of approximately 20×10^6 cells per flask and 25ml of growth medium were added after 96h incubation and the cells used as above.

The two seeding rates and feed times used in the small and large-scale protocols were designed to allow convenient timing of experiments. Both sets of protocols allowed efficient expression of activator(s).

(xi) Western blotting/immunogold silver staining

Following SDS PAGE, proteins were blotted onto nitrocellulose (NC) by electrophoretic transfer overnight at 4°C, 40V, 100mA in 25mM Tris/192mM Glycine/20% (v/v) Methanol pH 8.3. Unreacted sites on the NC were blocked with 5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) pH 7.2 for 1h, followed by a 2h incubation with rabbit anti-t-PA B chain IgG (48µg/ml) in 1% BSA/0.5% Tween 80/PBS pH 7.2 (diluent). The NC was washed, 3 x 5 mins, in 0.5% Tween 80/PBS pH 7.2 and then incubated for 1h in biotinylated secondary antibody (Janssen; 2µg/ml in diluent). The NC was washed as above and incubated in Auroprobe® BL plus streptavidin (Janssen) 1:100 in diluent for 2h. The NC was washed as above, followed by 1x1 min in deionised water, and silver enhanced using IntenSE® II kit (Janssen) for 15-20 min. All steps were carried out at ambient temperature with gentle shaking.

II. Identification of nucleotides, amino acid residues, N-termini, protein domains and chain nature in the examples

(i) Sequences

All t-PA numbering as in Pennica et al (1983) op. cit.; plasminogen amino acid numbering based on Sottrup-Jensen et al (1978) Atlas of Protein Sequence and Structure Vol. 5, Suppl. 3, p91, National Biomedical Research Foundation, Silver Spring, MD., but updated to include the extra amino acid residue indentified by Forsgren, M. et al (1987) FEBS Letters, 213, 254-260. Plasminogen nucleotide sequences as in Forsgren et al. (op. cit.). All urokinase numbering corresponds to Holmes et al., (1985) Bio Technology 3, 923-929.

(ii) N-termini

1. t-PA:

The mature N-terminus is normally believed to be equivalent to ser$_1$ (Pennica et al., 1983). Other forms also exist e.g. gly$_{-3}$ or val$_{+4}$ (Pohl et al., 1986 FEBS letters, 168 29-32).

2. plasminogen:

glu_1 indicat s the protein is believed to comprise the native (nascent) plasminogen N terminus i.e. amino acid residues 1 onwards.

lys_{78} indicates the protein is believed to comprise the processed lys_{78} N terminus. Alternative processed N-termini e.g. met_{69} and val_{79} are known in nature (Miyashita et al., 1988).

(iii) Protein Domains

The protein domains described in the examples have been abbreviated for convenience and are defined as follows:

1. t-PA:

F^t = amino acid residues 6 to 43 inclusive
G^t = amino acid residues 51 to 84 inclusive
B^t = amino acid residues 276 to 527 inclusive

2. plasminogen:

K_1^p = amino acid residues 84 to 162 inclusive
K_2^p = amino acid residues 166 to 243 inclusive
K_3^p = amino acid residues 256 to 333 inclusive
K_4^p = amino acid residues 358 to 435 inclusive
K_5^p = amino acid residues 462 to 541 inclusive

the 5 plasminogen
kringles K_1-K_5 = amino acid residues 84 to 541 inclusive

(iv) Chain nature
sc, indicates that the protein is in single chain form.
tc, indicates that the protein is in two chain form.

(v) Vectors
pTRE12 -(EP-0201153)-basic expression vector
pTRE15 -(EP-0201153)-encodes wild-type t-PA
pTRE24 -(EP-0207589)-encodes des(cys_{51}-asp87)t-PA
pTRE6F-(EP-0241208)-encodes t-PA finger domain

III. Construction and expression of hybrid proteins

The expression of the proteins of Examples 1 to 7 is carried out in Hela cells.

Example 1

Plasminogen 1-544/t-PA 262-527 (H204)

1.1 Construction of a plasmid carrying plasminogen cDNA

A plasminogen cDNA clone was obtained from a human liver cDNA library after screening with an oligonucleotide probe of sequence (A):
5' CC CCT CAC ACA CAT AAC AGG 3'   (A)
corresponding to nucleotides 49 to 68 of the sequence described in Malinowski et al Biochemistry 23, p4243 (1984).

DNA sequence of the portion of the plasminogen cDNA clone coding for the A chain of the protein was determined and two differences to the plasminogen cDNA sequence of Forsgren et al (1987) were noted.

The nucleotide at position 846 is T, the nucleotide at position 948 is G.

The 5' end of the clone was shown to correspond to nucleotide 27 of the sequence of Forsgren et al (1987).

Plasminogen cDNA was subcloned into the vector pUC8 (Vieira and Messing, 1982, Gene 19, 259) between the HincII and EcoRI sites to create plasmid pTRH6 Fig. 1a.

1.2 Modification of pTRE12

The single NarI site formerly in plasmid pTRE12 (EP-A-0 201 153) was destroyed by restriction cutting, blunt

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ending and religation forming pTRE12D Nar. Removal of the Narl site aided further manipulation.

1.3 Construction of a hybrid plasminogen: t-PA cDNA molecule (plasmid pTRH204)

(All plasminogen nucleotide numbering corresponds to Forsgren et al., 1987, op.cit.)   5
Three fragments were prepared by restriction digestion and agarose gel electrophoresis. These fragments
are as follows:-
   I: approximately 1.6kb: from HindIII plus AhaII digest (HindIII [5′ to the plasminogen coding sequence]
   to AhaII(1572)) of pTRH6.
   II: approximately 0.18 kb: from an AhaII plus DdeI digest (AhaII (1572) to DdeI (1748)) of pTRH6.   10
   III: approximately 5.3kb: largest fragment from a HindIII plus BglII digest, of pTRE12D Nar. This
   fragment carries pTRE12D Nar vector functions.
These fragments (I, II, III) were ligated with a kinased oligonucleotide linker (linker IV) comprising two
oligonucleotides of sequence:-
5′ TCAGTGTGCGGCGCCTGGTACCA 3′   (B)   15
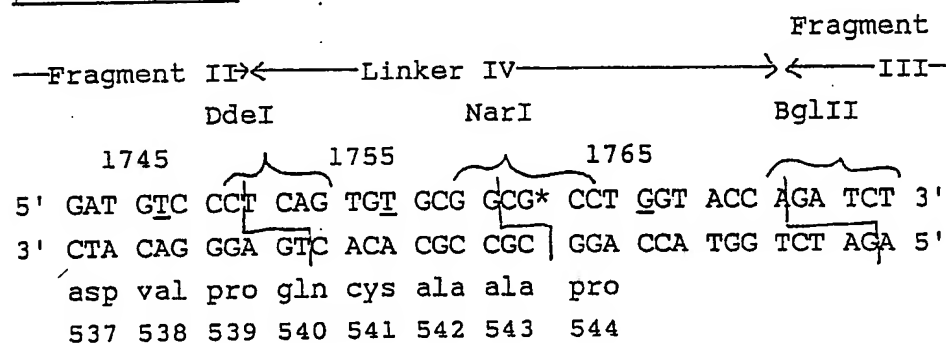5′ GATCTGGTACCAGGCGCCGCACAC 3′   (C)
After transformation into E.coli HB101 the plasmid pTRH9 (Fig. 2) was isolated.
The DNA in plasmid pTRH9 encodes most of the plasminogen-A chain, up to and including proline 544 i.e.
kringles I to 5; the DNA encoding the plasminogen B-chain and the remainder of the A-chain were absent. Use
of linker IV in construction of pTRH9 introduces a unique Narl site, as may be seen from Table I (which shows   20
the junction of fragments II and III and linker IV).

TABLE 1

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Junction of fragments II and III and linker IV in plasmid pTRH9.

```
                                                    Fragment        30
 —Fragment II><——————Linker IV——————————>←——————III—

            DdeI              NarI              BglII

      1745        1755            1765                      35
  5' GAT GTC CCT CAG TGT GCG GCG* CCT GGT ACC AGA TCT 3'

  3' CTA CAG GGA GTC ACA CGC CGC  GGA CCA TGG TCT AGA 5'

      asp val pro gln cys ala ala  pro                      40
      537 538 539 540 541 542 543  544
```

45

The plasminogen amino-acid sequence is shown
*This residue is C in plasminogen cDNA
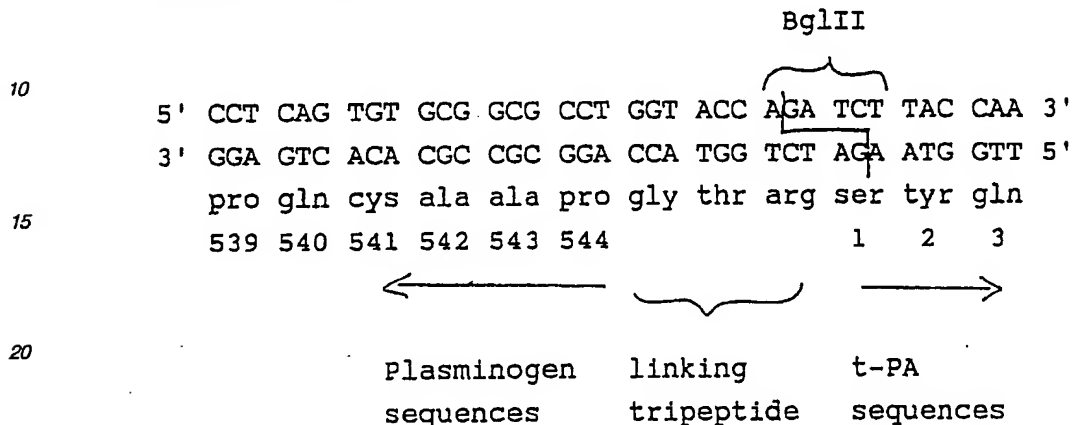Nucleotide numbering is included (above the sequence)
Plasmid pTRH9 was restricted with BglII and ligated with the 2kb BglII fragment encoding the mature t-PA
polypeptide isolated from PTRE15 (EP-A-0 201 153).   50
An E.coli HB101 clone carrying the t-PA insert in the same orientation (5′->3′) as the plasminogen 'A' chain
DNA was isolated; this plasmid was called pTRH20 (see Fig. 3).
Plasmid pTRH20 encodes a molecule comprising the five kringles of plasminogen (residues 1 to 544) linked
to the N-terminus of mature t-PA via the tripeptide gly-thr-arg. The junction of the plasminogen A-chain DNA
and t-PA A-chain DNA of plasmid pTRH20 is shown in Table 2.   55

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TABLE 2

Junction of the plasminogen A-chain DNA and t-PA A chain DNA in plasmid pTRH20.

BglII

```
5' CCT CAG TGT GCG GCG CCT GGT ACC AGA TCT TAC CAA 3'
3' GGA GTC ACA CGC CGC GGA CCA TGG TCT AGA ATG GTT 5'
    pro gln cys ala ala pro gly thr arg ser tyr gln
    539 540 541 542 543 544             1   2   3
```

Plasminogen   linking   t-PA
sequences   tripeptide   sequences

The DNA encoding the t-PA A-chain and part of the B-chain was removed from pTRH20 by restriction with NarI and SstI (located at t-PA nucleotide 1417 as described by Pennica et al 1983). The large SstI - NarI fragment (7.9kb) was isolated (fragment V).

An approximately 0.4kb BanII fragment (fragment VI) was isolated from PTRE15. The two relevant BanII sites are located at nucletotides 1024 and 1417 in the t-PA cDNA sequence described by Pennica et al (1983). BanII has a degenerate recognition site i.e.:

5' .... G Pu GC Py C.....3'
3' .... C Py CG Pu G.....5'

Note that the BanII site at 1417 is identical to the SstI site at 1417 and also that the sticky end created is compatible with an SstI sticky end. The 0.4kb BanII fragment encodes most of t-PA B-chain.

Two oligonucleotides (D) and (E):

```
5' CGCCGTCGACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATC
   AAAGGAGGGCT   3'                        (D)
```

```
5' CTCCTTTGATGCGAAACTGAGGCTGGCTGTACTGTCTCAGGCCGCAGGTC
   GACGG   3'                              (E)
```

were synthesized, kinased and combined to form linker VII.

Linker VII has a 5' NarI sticky end, compatible with the NarI sticky end of fragment V and a 3' BanII sticky end compatible with the BanII (nucleotide 1024) sticky end of fragment VI.
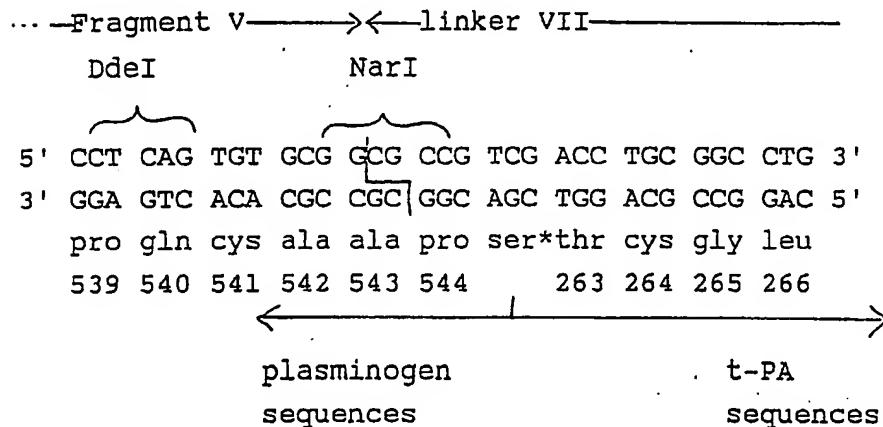
Fragments V and VI together with linker VII were ligated and transformed into E.coli HB101. A plasmid (pTRH204) with the structure depicted in Fig. 4 was isolated.

The junction of fragment V and linker VII is shown in Table 3.

Joining of fragments V and VI via the BanII (1417) and SstI sticky ends completed the reconstruction of the t-PA B-chain coding region.

TABLE 3

Junction of fragment V and linker VII in plasmid pTRH204

```
... —Fragment V——————>←—linker VII————————————
       DdeI              NarI

5'  CCT CAG TGT GCG GCG CCG TCG ACC TGC GGC CTG 3'
3'  GGA GTC ACA CGC CGC GGC AGC TGG ACG CCG GAC 5'
    pro gln cys ala ala pro ser*thr cys gly leu
    539 540 541 542 543 544     263 264 265 266
                    <——————————————————————————>

         plasminogen          . t-PA
         sequences              sequences
```

The plasminogen and t-PA amino-acid sequences at the junction are shown (plasminogen numbering 539-544 and t-PA numbering 263-266). The serine residue denoted * may be considered to be equivalent to plasminogen serine 545 or t-PA serine 262.

Plasmid pTRH204 was used to transfect human HeLa cells (as described in Methods) and produced a novel plasminogen activator (H204) believed to have the structure plasminogen 1-544/t-PA 262-527.

1.4 Alternative procedure for plasmid pTRH9

The plasminogen cDNA clone obtained from a human liver cDNA library after screening with oligonucleotide probe (A), is subcloned into the vector PTRE12 (EP-A-0 201 153) between the HindIII and BglII sites to create plasmid pTRH3 Fig. 1(b). The single NarI site formerly in plasmid PTRE12 is destroyed by restriction cutting, blunt ending and religation. Removal of the NarI site aids further manipulation.

Three fragments of pTRH3 are prepared by restriction digestion and agarose gel electrophoresis. These fragments are as follows:-

I: approximately 1.6kb: from a HindIII plus AhaII digest (HindIII [5' to the plasminogen coding sequence] to AhaII(1572)).

II: approximately 0.18kb: from an AhaII plus DdeI digest (AhaII (1572) to DdeI (1748)).

III: approximately 5.3kb: largest fragment from a HindIII plus BglII digest, carries the vector functions of pTRH3.

These fragments (I, II, III) are ligated with the kinased oligonucleotide linker IV described above.

Example 2

Plasminogen 1-544/t-PA 262-527(arg$_{275}$->gln)(H205)

Construction of plasmid pTRH205

A hybrid plasminogen activator molecule similar to that produced in Example I but differing only in the t-PA amino acid residue 275, which is glutamine in place of arginine, also forms part of this invention. The DNA coding for this plasminogen activator was prepared generally as described in Example 1, differing only in the nucleotide sequence of the linker in which the triplet coding for arg-275, CGC, in linker VII was replaced by CAG coding for gln-275. The resulting plasmid pTRH205 was used to transfect human HeLa cells (as described in Methods) and produced a novel plasminogen activator(H205) believed to have the structure plasminogen 1-544/t-PA 262-527 (arg$_{275}$->gln).

Example 3

Plasminogen 1-541/t-PA 262-527(H37)

Construction of plasmid pTRH37

Plasmid pTRH37 was constructed by ligating 2 fragments from pTRH204 (Example 1) together with fragment VI from PTRE15 and an oligonucleotide linker (linker XI):

Fragment IX : 7.2kb fragment of pTRH204 from the SstI site in the t-PA B chain coding region (nucleotide 1417 in the t-PA sequence) to the BstXI site in the plasminogen A chain coding region (nucleotide 1209 in the plasminogen sequence) (Fragment IX thus contains all the vector sequences).

Fragment X : 484 bp fragment of pTRH204 from the BStXI site (1209) to the AvaII site in the plasminogen A chain coding region at nucleotide (1693).

Fragment VI : (SstI-BanII) has been described previously (Example 1).

Linker XI was formed by annealing 4 oligonucleotides (H,I,J and K) of sequences:

5′ GTCCCTGGTG CTACACGACA AATCCGCGGA AACTTTACGA CTACTGTGAT GTCCCTCAGT GTTCGACCTG CGGCC 3′ (H)

5′ TGAGACAGTA CAGCCAGCCT CAGTTTCGCA TCAAAGGAGG GCT 3′ (I)

5′ CTCCTTTGAT GCGAAACTGA GGCTGGCTGT ACTGTCTCAG GCCGCAGGTC GAACACTGAGGG 3′ (J)

5′ ACATCACAGT AGTCGTAAAG TTTCCGCGGA TTTGTCGTGT AGCACCAGG 3′ (K)

A plasmid (pTRH37) was isolated which has the structure shown in Fig 5. The plasmid, when introduced into human HeLa cells, directed the expression of a novel plasminogen activator (H37) which has the same amino-acid sequence as protein H204 (Example 1) except for a deletion of the amino-acid residues alanine (542), alanine (543) and proline (544) immediately C-terminal to the Kringle 5 domain. The hybrid protein structure between Kringle 5 and the protease cleavage site is shown below using single letter notation:

541*   276
C̲STCGLRQYSQPQFR̲I̲

t-PA B chain

Residue 541 is the last cysteine in Kringle 5. The serine marked with an asterisk can be identified with $serine_{545}$ of plasminogen or $serine_{262}$ of t-PA.

Example 4

Plasminogen 78-544/t-PA 262-527(HOO)

4.1 Construction of 'lys78-cassette' plasmid

The lys78-cassette plasmid is a form of the plasmid pTRE12 containing a piece of synthetic DNA that can be used to convert a plasminogen clone from the glu_1 form (glu_1 is the normal secreted N terminus) to the lys_{78} form (lys_{78} is then the N terminal amino acid).

A pair of kinased oligonucleotide linkers (R+S and T+V) comprising four oligonucleotides:

5′ AGCTTCCAGT CCCAAAATGG AACATAAGGA AGTGGTTCTT CTACTTCTTT TATTTCTGAA ATCGGGACAA GGAAAAGTGT ATCTCT 3′ (R)

5′ ACACTTTTCC TTGTCCCGAT TTCAGAAATA AAAGAAGTAG AAGAACCACT TCCTTATGTT CCATTTTGGG ACTGGA 3′ (S)

5′ C AGAGTGCAAG ACTGGGAATG GAAAGAACTA CAGAGGTACC ATGTCCAAAA CAAAAAATGG CATCACCTGT CAA 3′ (T)

5′ GATCTTGACA GGTGATGCCA TTTTTTGTTT TGGACATGGT ACCTCTGTAG TTCTTTCCAT TCCCAGTCTT GCACTCTGAG AGAT 3′ (V)

were prepared and ligated with the large HindIII-BglII fragment from PTRE12.

The resulting plasmid contained a region formed by the oligonucleotides comprising nucleotides 65-132 inclusive, joined to nucleotides 364-450 inclusive of native plasminogen. These nucleotides encode the signal sequence of 19 amino-acids as described by Forsgren et al, (1987) and amino acids 78-106 inclusive of the plasminogen A chain. A unique Kpn1 site was introduced (Table 4). The structure of the oligonucleotide insert

was verified by DNA sequencing.

Table 4

Structure of the lys$_{78}$-cassette insert

```
                   met glu his lys glu val val leu leu
AGCTTCCAGTCCCAAA ATG GAA CAT AAG GAA GTG GTT CCT CTA
Hind AGGTCAGGGTTT TAC CTT GTA TTC CTT CAC CAA GAA GAT
III

leu leu leu phe leu lys ser gly gln gly*lys val tyr
CTT CTT TTA TTT CTG AAA TCA GGT CAA GGA AAA GTG TAT
GAA GAA AAT AAA GAC TTT AGT CCA GTT CCT TTT CAC ATA


leu ser glu cys⁺lys thr gly asn gly lys asn tyr arg
CTC TCA GAG TGC AAG ACT GGG AAT GGA AAG AAC TAC AGA
GAG AGT CTC ACG TTC TGA CCC TTA CCT TTC TTG ATG TCT


gly thr met ser lys thr lys asn gly ile thr cys gln
GGT ACC ATG TCC AAA ACA AAA AAT GGC ATC ACC TGT CAA BglII
CCA TGG TAC AGG TTT TGT TTT TTA CCG TAG TGG ACA GTT CTAG
KpnI                                 SfaNI
```

The HindIII and BglII sticky ends were used to clone into the HindIII-BglII sites in PTRE12. SfaNI cuts at a site remote to its recognition sequence. gly* indicates the last residue of the signal sequence; cys⁺ is the first residue of the K$_1$P domain.

4.2 Construction of plasmid pTRH34 (Plasminogen 1-561/t-PA 276-527)

Plasmid pTRH34 was constructed essentially as described for plasmid pTRH204 (Example 1) by ligation of fragments V and VI together with a linker, but in place of linker VII, linker VIII was used. Linker VIII was formed by annealing 2 oligonucleotides (F and G):
5′ CGCCTTCATT TGATTGTGGG AAGCCTCAAG TCGAGCCGAA GAAATGTCCC GGGAGGATCA AAG-
GAGGGCT 3′   (F)
5′ CTCCTTTGAT CCTCCCGGGA CATTTCTTCG GCTCGACTTG AGGCTTCCCA CAATCAAATG AAGG
3′   (G)
A plasmid pTRH34, with the structure shown in Fig. 6 was isolated. Plasmid pTRH34 when used to transfect human HeLa cells directs the synthesis of a novel plasminogen activator comprising the entire plasminogen A chain (glu$_1$-arg$_{561}$) linked directly to the complete t-PA B chain (ile$_{276}$-pro$_{527}$).

4.3 Construction of plasmid pTRH00

Plasmid pTRHOO is a form of the plasmid pTRH204 (Example 1) in which the nucleotide sequence coding for amino acids glu$_1$-lys$_{77}$ of the plasminogen protein has been deleted.
The following DNA fragments were prepared by restriction endonuclease digestion and agarose gel electrophoresis:-
Fragment I :-   approximately 160bp; from a HindIII plus SfaN1 digest of the lys$_{78}$-cassette plasmid of Example 4.1 [i.e. from the HindIII site at the 5′ end of the oligonucleotide linker sequence to the internal SfaN1 site - see Table 4] and carries a nucleotide sequence coding for the signal sequence of 19 amino-acids as

described by Forsgren et al (1987) joined to amino acids 78-104 inclusive (the SfaN1 overhang extends to amino-acids 105 and 106) of the plasminogen A chain.

Fragment II :- approximately 772bp; from a SfaN1 plus BstXI digest of pTRH34 of Example 4.2 (SfaN1(437) to BstXI(1209)), this carries most of K1^p, all of K2^p and K3^p and the N-terminal section of K4^p.

Fragment III:- approximately 7Kb; from a BstXI and HindIII digest of pTRH204 (BstXI cuts at (1209) and HindIII is located 5′ to the plasminogen coding sequence). This carries most of K4^p, all of K5^p, B^t and the vector functions of PTRE12.

These fragments were ligated, after transformation into E.coli HB101 the plasmid pTRH00 was isolated (Fig. 7). pTRH00 was used to express the novel hybrid plasminogen activator HOO in HeLa cells.

Example 5

t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-pA 262-527 (HO1)

Plasmid pTRH01 was designed to encode a mature protein in which the F^t coding domain from t-PA is joined to the 5′ end of the K1^p coding domain in plasmid pTRH00 (Example 4); the gross domain structure of the encoded native protein can be abbreviated as below:

F^{t}K$_1$^{p}K$_2$^{p}K$_3$^{p}K$_4$^{p}K$_5$^{p}B^t

Construction of plasmid pTRH01

Two DNA fragments were prepared by restriction endonuclease digestion and agarose gel electrophoresis. These fragments were as follows:-

Fragment I :- approximately 360bp: from a HindIII plus BamHI digest of plasmid pTR6F (disclosed in EP-0241208). The insert in pTR6F carries the 5′ untranslated region and the signal/pro regions as well as the F^t domain.

Fragment II :- approximately 8kb: from a HindIII plus KpnI digest of plasmid pTRH00 (Example 4); this carries almost all of K1^p, all of K2^p, K3^p, K4^p,K5^p, B^t and the vector functions of pTRE12.

These fragments were ligated with a kinased oligonucleotide linker comprising two oligonucleotides (W and X) of sequence:-

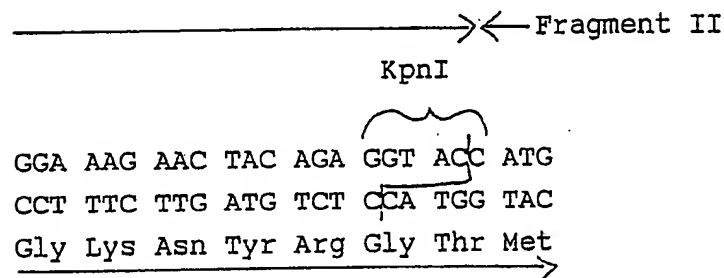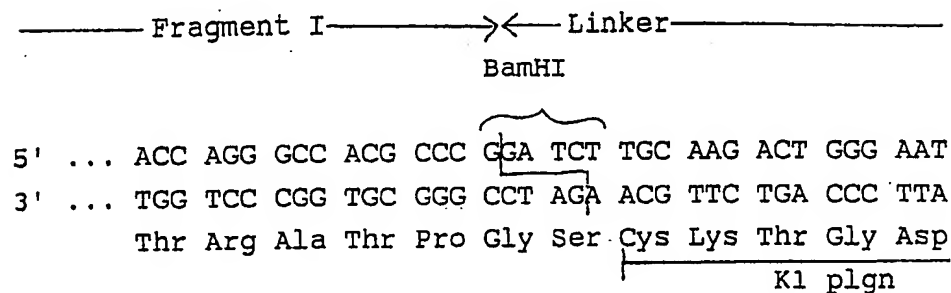5′ GATCTTGCAAGACTGGGAATGGAAAGAACTACAGAGGTAC 3′ (W)
5′ CTCTGTAGTTCTTTCCATTCCCAGTCCTGCAA 3′ (X)

After transformation into E.coli the plasmid PTRH01 (Fig. 8) was isolated. DNA sequencing confirmed the structure of the F^t/K1^p join (Table 5).

pTRH01 was used to express a novel hybrid plasminogen activator (HO1), believed to have the sequence t-PA 1-50/t-PA88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527, in HeLa cells.

Table 5

Junction of Fragments I and II and linker (W + X)
in plasmid pTRH01

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```
————————— Fragment I—————————>←——Linker——————————————
                           BamHI

5' ... ACC AGG GCC ACG CCC GGA TCT TGC AAG ACT GGG AAT
3' ... TGG TCC CGG TGC GGG CCT AGA ACG TTC TGA CCC TTA
       Thr Arg Ala Thr Pro Gly Ser·Cys Lys Thr Gly Asp
                                                  K1 plgn
```

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```
——————————————————————————————————>←——Fragment II
                KpnI

GGA AAG AAC TAC AGA GGT ACC ATG
CCT TTC TTG ATG TCT CCA TGG TAC
Gly Lys Asn Tyr Arg Gly Thr Met
——————————————————————————————————————>
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Example 6

t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (HO2)

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   Plasmid pTRH02 was designed to encode a mature protein in which the F^t and G^t coding domains from t-PA are joined to the 5' end of the K_1^P coding domain in plasmid pTRH00 (Example 4); the gross domain structure of the encoded native protein can be abbreviated as below:
   $F^tG^tK_1^PK_2^PK_3^PK_4^PK_5^PB^t$

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6.1 Preparation of DNA encoding the t-PA finger and growth factor domains (plus the t-PA signal/pro and 5' untranslated regions)

   6μg of the plasmid PTRE15 (described in EP-A-O 201 153) was digested with MaeII. The MaeII cut DNA was flush-ended and kinased BamH1 linkers (0.1 A_{260} units; Amersham DC1203) of sequence 5'CCCGGATCCGGG3' were ligated to 5μg of the flush-ended MaeII fragments. After ligation excess linkers were removed and cohesive 'sticky-ends' created by digestion with BamHI. The BamH1 digest was electrophoresed on a 1% low melting point agarose gel. On this gel there was a group of three bands with mobility between that of the 1353 and 872 base pair fragments found in a lambda DNA-HindIII/ X174RF-HaeIII digest (Drigest TMIII, Pharmacia). The largest of these three bands, approximately 1200bp, was recovered (no special attempt was made to ensure that the other bands of similar mobility did not contaminate this preparation, since undesired molecules were eliminated in the cloning strategy). The 1200bp fragment was digested with HindIII to create the desired approx. 470bp fragment with a HindIII 5' sticky end and a 3' BamHI sticky end (converted from the MaeII site).

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This finger-plus-growth-factor domain encoding DNA was sub-cloned into plasmid pTRE12 (EP-A-0207589) between the HindIII and BamHI sites to create plasmid pTR6FG. PTR6FG also carries the 5' untranslated region and signal/pro regions.

6.2 Construction of pTRH02

Two DNA fragments were prepared by restriction endonuclease digestion and agarose gel electrophoresis. These fragments were as follows:

Fragment I : approximately 470bp: from a HindIII plus BamHI digest of plasmid pTR6FG.

Fragment II : approximately 8kb: from a HindIII plus KpnI digest of plasmid pTRH00 (Example 5), carries almost all of K1^p, all of K2^p, K3^p, K4^p, K5^p, B^t and the vector functions of PTRE12.

These fragments were ligated with a kinased oligonucleotide linker comprising two oligonucleotides (W) and (X) of Example 5.

After transformation into E.coli the plasmid pTRH02 was isolated (Fig. 9).

Plasmid pTRH02 was used to express a novel hybrid plasminogen activator (HO2), believed to have the sequence t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527, in HeLa cells.

Example 7

Plasminogen 1-546 /u-PA 137-411(H25)

Construction of plasmid pTRH25

A human urokinase cDNA clone is identified by oligonucleotide probing and is isolated by conventional means (Holmes et al, (1985) Bio Technology 3, 923-929). The cDNA clone is modified by conventional in vitro mutagenesis techniques to incorporate an SstI site at nucleotide 563 and a BglII site in the 3' untranslated region. Amino acids 143 and 144 are then coded by the nucleotide triplets GAG CTC rather than the native GAA TTA (plasmid pTRU2).

To create the hybrid enzyme coding sequence, plasmid pTRH9 (Example 1) is digested with NarI and BglII and the approximately 7kb fragment I (carrying vector functions and most of the plasmin A chain coding region) is isolated. Plasmid pTRU2 is digested with SstI and BglII and the urokinase B-chain coding fragment II isolated. An oligonucleotide linker III composed of two kinased oligonucleotides Y and Z is made.

5' CG CCT TCA TTT CCC TCC TCT CCT CCA GAA GAG CT 3'

(Y)

3'   GGA AGT AAA GGG AGG AGA GGA GGT CTT C 5' (Z)

Fragments I and II and linker III are ligated and transformed into E. coli HB101 and plasmid pTRH25 isolated (Fig.10). The new plasmid encodes a novel hybrid enzyme. The structure in the region of the join between fragment I, II and linker III is believed to be as shown in Table 6.

Table 6

Nar I

```
5' ... TGT GCG GCG CCT TCA TTT CCC TCC TCT CCT CCA GAA
3' ... ACA CGC CGC GGA AGT AAA GGG AGG AGA GGA GGT CTT
       cys ala ala pro ser phe pro ser ser pro pro glu
       541 542 543 544 545 546 137 138 139 140 141 142
```

plasminogen sequences   urokinase sequences.

Sst I

```
GAG CTC AAA ...
CTC GAG TTT ...
glu leu lys
143 144 145
```

Plasmid pTRH25 is used to transfect human Hela cells and produce a novel plasminogen activator.

Example 8

Expression of plasminogen 1-544/t-PA 262-527 (H204) in Chinese Hamster Ovary cells

The plasmid pSV2dhfr was obtained from the American Type Culture Collection (ATCC 37146 : Molec. Cell. Biol. 1: 854-864, 1981). The plasmid BPV-MT-Xho was obtained from D. Hamer (National Institutes of Health, Bethesda, Maryland) and contains a version of the mouse metallothionein-I gene (Hamer and Walling (1982) J. Mol. Appl. Genet. 1, 273-288) in which the BglII site just 5' to the translation start point has been converted to an XhoI site by linkering. The t-PA mutein-plasmid pTRE24 has been described previously (EP 0 207 589).

8.1 Construction of an amplifiable expression vector:

a. Construction of pTRH69

The plasmid pTRH69 was constructed from two fragments A and B, isolated from the plasmid PSV2dhfr and the plasmid BPV-MT-XhoI respectiely.
Fragment A:- The plasmid PSV2dhfr (Fig.11) was linearised with EcoRI, blunt ended by infilling and linkered with XhoI linkers. The linearised plasmid was then digested with XhoI and BglII releasing fragment A, (a 3.4kb fragment encoding the dihydrofolate reductase cDNA and SV40 early promoter sequences).
Fragment B:- The plasmid BPV MT-XhoI was linearised with SstI, blunt-ended by nuclease digestion and linkered using BglII linkers. The linear plasmid was then digested with HindIII, blunt ended by infilling and linkered with XhoI linkers. The plasmid was then digested with BglII and XhoI releasing fragment B (0.3kb encoding the 3' metallothionein polyadenylation sequences) (Fig. 12).
Fragment A and B were isolated by LMP agarose electrophoresis and ligated together. The ligated DNA was tranformed into E.coli HB101 and the plasmid PTRH69 was obtained (Fig. 13).

b. Construction of pTRH71

The plasmid pTRH69 was linearised with XhoI and phosphatased. The LMP agarose-purified fragment was

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ligated with a 3.3Kb XhoI fragment derived from the plasmid pTRE24 which encodes a modified t-PA protein. The ligated DNA was transformed into E.coli HB101 and the plasmid pTRH71 was obtained (Fig. 14).

c. Construction of pTRH11

The plasmid pTRH71 was digested with MluI and BamHI; a 4.5Kb fragment (comprising the vector functions) was isolated by LMP agarose gel purification and ligated with a 4.1Kb MluI/BamHI fragment isolated from pTRH204 (Example 1) encoding hybrid protein H204. The ligated DNA was transformed into E.coli HB101 and the plasmid pTRH11 was obtained (Fig. 15).

8.2 Expression of hybrid protein

a. Cell preparation

CHO cells were trypsinised and plated out at 5×10^5 per 60mm dish and left in growth medium (Hams F12 nutrient media (041-1765) with 1% stock glutamine (043-5030), 1% stock penicillin/streptomycin (043-5070) and 10% bovine foetal calf serum (013-6290); Gibco, Paisley, Scotland) at 37°C in a humidified incubator in an atmosphere of 5% CO_2/95% air. After 18hrs the cells were re-fed, after a further 3hrs the cells were used for DNA transfection.

b. Transfection procedure

The transfection procedure, carried out in growth medium used calcium coprecipitation and glycerol shock as described in DNA Cloning Ed. D.M. Glover (Chap. 15, C. Gorman). Following transfection the cells were maintained in growth medium for 46hrs under growth conditions (as above) prior to the selection procedure.

c. Selection and Co-amplification

The selection and co-amplification procedure was carried out essentially as described by R.J. Kaufman. (1985) Molecular and Cellular Biology 5, 1750-1759. 46hrs post transfection the cells were medium changed into selective medium (Eagles MEM (041-1095) with 1% stock sodium pyruvate (043-1360), 1% stock glutamine (043-5030), 1% stock penicillin/streptomycin (043-5070) 0.35% proline (22mg/ml) and 10% dialysed bovine foetal calf serum (063-6300) (Gibco, Paisley, Scotland). The cells were maintained in selective medium for 8-10 days until colonies of dhfr⁺ cells appeared. When colonies were established the cells were medium changed into selective medium containing methotrexate, (A6770; Sigma, England). After 6 days in selective medium containing methotrexate, the selective medium was modified by replacing the Eagles MEM and sodium pyruvate components with α MEM (041-02561). The methotrexate concentration was initially 0.02μM and was increased stepwise to 0.05μM.

d. Detection of hybrid protein H204

During the amplification procedure aliquots of growth medium from growing cells were assayed for plasminogen activator production by fibrin plate and zymography as described by Dodd et al. (1986) Thrombosis and Haemostasis, 55, 94-96. Zymography revealed a fibrinolytically active protein with apparent M_r approximately 100,000, which is consistent with the expected molecular weight of hybrid protein H204.

IV. Purification and post-synthetic modification of hybrid proteins

Example A

Purification of plasminogen 1-544/t-PA 262-527 (H204)

200ml conditioned harvest media obtained from HeLa cell cultures transfected with the plasmid PTR H204 (Example 1) was centrifuged at 9000g for 30 min and the supernatant retained. It was applied to a column (i.d., 90 mm; h. 220 mm) of Sephadex G25[1]equilibrated in PBS (Dulbecco A)/0.01% Tween 80 pH 7.4 (PBS/TW). The first approx. 0.35 vt was discarded. The following 500ml was retained and was chromatographed on zinc chelate-Sepharose (Vt = 80ml) and lysine Sepharose (Vt = 20ml) essentially as described previously (Dodd, I. et al (1986a) FEBS Lett 209 13). The major difference was that the purified activator was dissociated from the second column using 0.05M sodium phosphate/0.1m sodium chloride/0.01% Tween 80 pH 7.4 (PST) containing 0.02M ε-aminocaproic acid (ε-ACA). Active fractions eluted by the PST/ε-ACA buffer were identified using the chromogenic substrate H-D-ile-pro-arg-p nitroailide (Dodd, I. et al (1986b) Thromb. Haem.

[1] Sephadex and Sepharose are Trade Marks

55 94) and were concentrated by stirred-cell ultrafiltration (YM10, Amicon). The ultrafiltered retentate (2.0ml) contained 14400 IU and 4800 SU (Dodd, I. et al (1986b). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) followed by fibrin zymography (Dodd, I. et al. 1986b) showed that the product contained a major fibrinolytic species with apparent M_r approximately 100000, consistent with the isolation of a glu_1 form of the hybrid activator.

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Example B

Synthesis of lys_{78} tc H204 protein (two chain plasminogen 78-544/t-PA 262-527)

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A preparation of purified H204 protein from Example A that had been buffer-exchanged into PST/10mg ml^{-1} mannitol/50μm ε-ACA (89,000 IU, 1.9 ml) was treated with a 0.01 fold molar excess of plasmin at 25°C for 16h and then stored at -40°C. Subsequent analysis of the product showed that it contained 68,000 IU and 32,000 SU. The activator had an apparent M_r of approximately 90,000 by SDS PAGE followed by fibrin zymography. *15* The lys,tc nature of the product was confirmed by SDS PAGE followed by immuno blotting using a polyclonal antibody against the t-PA B chain (see Methods section).

Example C

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Preparation of 2-Amino-4-chlorobenzoyl plasminogen 78-544/t-PA 262-527 (two chain) ex.Hela cells

Purified lys_{78}-tc H204 protein from Example B (3900 SU) in PST/12.5μM ε-ACA/2.5mg ml^{-1} Mannitol (1ml) was treated with a 5mM solution of 4-amidinophenyl-2'-amino-4'-chlorobenzoate hydrochloride (Example 11 *25* hereinafter) in dimethylsulphoxide (2μl, 20eq). After 1h incubation at 25°C an inhibition of 40% was noted. A further aliquot (4μl, 40eq) of acylating agent solution was added and after a further 1h at 25°C 93% inhibition had been obtained. The material was buffer exchanged using a Sephadex G25 (PD 10) column into PST/166μM ε-ACA/1.66mg ml^{-1} mannitol (1.35ml) and frozen at -40°C.

The material was deacylated by dilution of stock solution (0.3ml) with PST (0.2ml) to give a final solution of *30* PST/100μM ε-ACA/1mg ml^{-1} mannitol. The deacylation rate in the above buffer system at 37°C was 1.05×10^{-4} sec^{-1} i.e. a $t_{1/2}$ of 110 min.

Example D

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Purification of Plasminogen 1-544/t-PA 262-527 ($arg_{275}->gln$)(H205)

515ml conditioned harvest medium obtained from HeLa cultures transfected with pTR H205 (Example 2) was purified essentially as described in Example A. The hybrid activator was eluted from the lysine Sepharose *40* column using PST/20mM ε-ACA, was concentrated by ultrafiltration and was then buffer-exchanged into PST/10mg ml^{-1} mannitol/50μm ε-ACA (2.0ml). The product contained 18,000 IU and 6600 SU. Analysis by SDS PAGE followed by fibrin zymography showed the preparation contained two major fibrinolytic species, at apparent M_r approximately 100,000 and 90,000. These are believed to be the glu_1 and lys_{78} forms of the hybrid activator.

45

Example E

Purification of plasminogen 1-541/t-PA 262-527 (H37)

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510ml clarified, conditioned harvest medium that had been obtained from HeLa cell cultures that had been transfected with the plasmid PTR H37 (Example 3) was purified essentially as described for H204 in Example A. The hybrid activator was dissociated from the lysine Sepharose column using PST/20mM ε-ACA, was concentrated by ultrafiltration and was then buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ε-ACA *55* (2.0ml). The product contained 36,000 IU and 15,000 SU. Analysis by SDS PAGE followed by fibrin zymography showed the preparation contained two major fibrinolytic species, at apparent M_r of approximately 100,000 and 90,000. These are believed to be the glu_1 and lys_{78} forms of the activator. SDS PAGE followed by silver staining of non-reduced activator showed the preparation contained major protein bands at M_r approximately 90,000.

Analysis by SDS PAGE followed by immunoblotting using a polyclonal antibody against the t-PA B chain *60* supported the zymography results indicating the presence of glu_1 and lys_{78} forms, and, in addition, showed that the product was all two-chain.

65

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Example F

Preparation of lys$_{78}$ tc H37 protein (two chain plasminogen 78-541/t-PA 262-527)

Purified H37 protein (0.28ml, 7400 SU/ml) prepared as described in Example E, containing equal amounts of the glu$_1$ and lys$_{78}$ forms, was treated either with or without an approx. 0.03 fold molar excess of plasmin at 37°C for 17h. The amidolytic activity (SU) of both products was the same as that of a control preparation that had been stored at -40°C. Analysis by SDS PAGE followed by fibrin zymography showed that the plasmin-treated H37 protein, and, to a lesser extent, the non-plasmin treated H37 protein, had been converted to the lys$_{78}$ form.

Example G

Synthesis of 2-amino-4-chlorobenzoyl-plasminogen 1-541/t-PA 262-527 (two chain) and -plasminogen 78-541/t-PA 262-527 (two chain)

Purified H37 protein from Example F (10nmoles) in PST/10mg ml^{-1} mannitol/50μM ε-ACA (2.0ml) was treated with 4-amidinophenyl-2'-amino-4'-chlorobenzoate.HCl (2μmoles, 40μl) at 25°C. After 60 min the amidolytic activity of the preparation had decreased to <2% of the original activity. The material was buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ε-ACA (3.0ml) and stored at -40°C.

An aliquot of the acylated product was later diluted with 5 volumes PST/10mg ml^{-1} mannitol/50μM ε-ACA and allowed to deacylate at 37°C.

Example H

Purification of plasminogen 78-544/t-PA 262-527 (H00)

500ml conditioned harvest medium obtained from HeLa cell cultures transfected with the plasmid pTR H00 (Example 4) was clarified by centrifugation (9000g/30min) and was purified as described for H204 protein in Example A. The hybrid activator was eluted from the lysine Sepharose column (Vt, 49ml) using PST/20mM ε-ACA, was concentrated by ultrafiltration and then buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ε-ACA (2.1ml). The product contained 5,700 IU and 2,700 SU. SDS PAGE followed by fibrin zymography indicated the presence of a major fibrinolytic species with apparent M$_r$ of approximately 90,000. SDS PAGE followed by staining for protein or immunoblotting using polyclonal antibody against the t-PA B-chain revealed the material was in the two-chain form. These results are consistent with the isolation of lys$_{78}$ tc hybrid plasminogen activator.

Example I

Purification of t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (H02)

380ml Conditioned harvest medium obtained from HeLa cell cultures transfected with the plasmid pTR H02 (Example 6) was clarified by centrifugation (9000g/30min) and purified essentially as described for H204 protein in Example A. The hybrid activator was dissociated from the lysine Sepharose column (Vt, 55ml) using PST/20mM ε-ACA. The eluate was concentrated by ultrafiltration and then buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ε-ACA (2.0ml) to give the product. This contained 42,000 IU and 20,400 SU. SDS PAGE followed by fibrin zymography showed a major fibrinolytic species at M$_r$ of approximately 100,000. SDS PAGE followed by protein staining or immunoblotting using a polyclonal antibody against the t-PA B-chain showed that the hybrid enzyme was in the two-chain form.

Example J

Synthesis of 2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (two chain)

H02 protein (1ml, 0.25 nmoles) purified as described in Example I was treated with 4-amidinophenyl-2'-amino-4'-chlorobenzoate.HCl (1.25μl, 25nmoles) at 25°C. After 1h 93% of the amidolytic (S2288) activity of the preparation had been lost. After addition of another 25nmoles acylating agent and a further incubation for 15min the material was buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ε-ACA (2.2ml), aliquoted and stored at -70°C.

Subsequently, an aliquot of the product was diluted with 1 volume 0.1M Tris/0.15M NaCl/20% (v/v)

glycerol/0.01% Tween 80 pH 7.4 and allowed to deacylate at 37°C. The rate of deacylation was determined by assaying the S2288 activity of the solution. The deacylation rate constant of the acylated protein under the described conditions was $3.9 \times 10^{-4} \mathrm{sec}^{-1}$.

Example K   *5*

Purification of t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (H01)

2.0ml conditioned harvest medium from HeLa cell cultures transfected with the plasmid pTR H01 (Example   *10*
5) was buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ε-ACA (3.0ml) and concentrated by ultrafiltration
(Centricon[2]10, Amicon) to about 0.45ml. The concentrate contained 520 IU/ml; analysis by SDS PAGE
followed by fibrin zymography revealed a major fibrinolytic species at apparent M_r approx. 100000.
The hybrid activator was purified by incubating the 0.45ml solution with 0.2ml lysine Sepharose suspension,
removing the supernatant, washing the gel with PST and finally eluting the adsorbed activator with PST/20mM   *15*
ε-ACA, to yield about 0.8 ml solution containing 60 IU/ml partially-purified H01 protein.

Example L   ·

                                                                                                                        · *20*

Purification of plasminogen 1-544/t-PA 262-527(H204) produced by Chinese Hamster Ovary cells.

CHO cells transfected with pTRH11 (Example 8.1c.) and selected for survival in methotrexate at 0.05 μM   ·
(Example 8) were harvested (25ml per 175cm^2 flask) for 24h in αMEM (041-02561) containing 1%
penicillin/streptomycin (043-5070), 1% glutamine (043-5030) (Gibco, Paisley, Scotland) and 3mM sodium   *25*
butyrate (9686730F; BDH Chemical Ltd., Poole, England). 1400ml of harvest medium was collected.
The harvest medium was clarified by centrifugation (9000g/30 min) and purified on zinc chelate Sepharose
(Vt, 230 ml) and lysine sepharose (Vt, 46 ml) using essentially the same method as that described for protein
H204 in Example A. The major difference was that the lysine Sepharose column was not eluted with the ε-ACA-
containing buffer, but with the following, in succession: *30*
    a. 0.02MTris/0.5M NaCl/0.01% Tween 80 pH 7.4
    b. as a. but also containing 0.1M L-arginine
    c. as a. but also containing 0.2M L-arginine
    d. as a. but also containing 0.3M L-arginine
    e. as a. but also containing 0.4M L-arginine   *35*
    f. as a. but also containing 0.5M L-arginine
Analysis of the individual fractions that had been collected showed that both endogenous CHO-cell t-PA
and protein H204 had adsorbed to the lysine Sepharose column, and both had been dissociated by the
arginine - containing buffers. Analysis by SDS PAGE followed by fibrin zymography further showed that the
majority of purified protein H204 had clearly been separated from the endogenous CHO cell t-PA, the latter   *40*
being dissociated by a lower concentration of arginine. The apparent M_r of H204 by SDS PAGE followed by   ·
fibrin zymography was approx. 100,000 (with a minor species at approx. 90000), clearly different from the
endogenous CHO t-PA, at M_r approx. 65000.
Further analysis of H204 protein by Western blotting using a polyclonal antibody against t-PA B-chain
showed that the bulk of the protein was in the two chain form.   *45*

Example M

Synthesis of 2-amino-4-chlorobenzoyl plasminogen 1-544/t-PA 262-527 and 2-amino-4-chlorobenzoyl   *50*
plasminogen 78-544/t-PA 262-527 ex. CHO cells

10,000 IU purified two-chain H204 protein from CHO cells (Example L) in PsT/10mgml^{-1} mannitol/50μM
ε-ACA (1ml) was treated with 4-amidinophenyl-2'-amino-4'- chlorobenzoate.HCl (20mM, 2.5μl) for 1h at 25°C
followed by an additional 2.5μl aliquot for a further 20 min. The acylated H204 was buffer-exchanged into   *55*
PST/10mgml^{-1} mannitol/50μM ε-ACA (1.5ml) using Sephadex G25 and aliquoted. Fibrin plate assay indicated
that the acyl product contained 3600 IU/ml. The deacylation rate constant was determined as $1.2 \times 10^{-4} \mathrm{sec}^{-1}$.
Analysis of the acylated product by SDS PAGE followed by fibrin zymography showed two definite species,
with apparant M_r approximately 100000 and 90000. The former is likely to be the glu$_1$ form of acylated H204. The
latter is most likely the lys$_{78}$ form.   *60*

V. Preparation of derivatives of the invention

                                                                                                                        65

[2] Centricon is a Trademark

Methods for Examples 9-17

(a) Chromogenic substrate assay

Urokinase and t-PA were assayed against the chromogenic substrates (KabiVitrum, Sweden) S-2444 and S-2288, respectively at a substrate concentration of I mM in 0.1 M triethanolamine.HCl pH 8.0 at 25°C. An SU is defined as the amount of activity that gives an O.D. increase of 0.001/min in 0.5 ml substrate in a 1 cm pathlength cell.

(b) Rate constant determinations

The pseudo first order rate constant is determined by hydrolysing the acyl-enzyme under physiological conditions, i.e. in isotonic aqueous media at pH 7.4 and at 37°C. At regular intervals aliquots are withdrawn and incubated with a chromogenic substrate and the rate of conversion of the substrate measured as indicated above.

The hydrolysis is followed until such time as the rate of conversion of substrate reaches a maximum. The rate constant k may then be calculated by plotting:

$\text{Log}_e (1-A_t/A_{max})$ against t

where A_{max} is the maximum rate at which an aliquot converts substrate and A_t is the rate at which an aliquot converts substrate at time t.

Preferably such rate constants are calculated by computerised non-linear regression analysis, fitting the A_t and time data to the equation:

$A_t = A_o + (A_{max} - A_o) (1 - e^{-kt})$

where A_o is the activity of the acyl-enzyme preparation before deacylation.

Example 9

4'-Amidinophenyl 2-amino-4-fluorobenzoate

(a) 2-Methyl-5-fluoroacetanilide

5-fluoro-2-methylaniline (6.26g, 50mmol) and triethylamine (7.26g, 10ml, 72mmol) were dissolved in dichloromethane (50ml). This mixture was added dropwise to an ice-cooled solution of acetyl chloride (3.925g, 3.55ml, 50mmol) in dichloromethane (100ml) under an atmosphere of nitrogen. The solution was allowed to warm to room temperature and stirred for 3h. The organic layer was then washed successively with 10% sodium hydrogen carbonate solution (50ml), 2M hydrochloric acid (50ml) and saturated sodium sulphate solution (50ml). The organic layer was dried, filtered and evaporated and the residual solid purified by column chromatography (70g silica in dichloromethane -> 10% ethyl acetate/90% dichloromethane) to leave the title compound (5.41g, 65%). m.p: 127°C ex ethanol and water

^{1}H nmr (CDCl$_3$) δ: 6.5-7.8 (4H, m, aryl-$\underline{H}$ + N-$\underline{H}$), 2.15 (6H,s,C$\underline{H}_3$ + COC$\underline{H}_3$)

IR v_{max} (Nujol): 3270, 1650, 1600, 1530, 1285, 1250, 860, 800 and 610cm^{-1}

Found: C 64.40 H 6.26 N 8.27

C$_9$H$_{10}$NOF

requires: C 64.66 H 6.03 N 8.27%.

(b) 2-(Methylcarbonylamino)-4-fluorobenzoic acid

2-Methyl-5-fluoroacetanilide (Example 9(a)) (2.0g, 10mmol) was dissolved in water (50ml) and potassium permanganate (2.25g, 14mmol) was added. The reaction was stirred and heated to reflux until the purple colour disappeared. Two more aliquots of the oxidant (2 x 1.125g) were added at approximately 2h intervals.

The reaction was allowed to cool after 6h and the solution was brought to basic pH. It was then filtered through celite and the aqueous solution was acidified. The aqueous layer was extracted with ethyl acetate (50ml) which was then dried, filtered and evaporated to leave the title compound (0.5g, 22%). m.p: (sublimes above 140°C)from ethanol and water

^{1}H nmr (CDCl$_3$) δ: 11.4 (1H,br s, CO$_2$H), 8.2 (2H, m, aryl-$\underline{H}$), 6.8 (1H, m, aryl-$\underline{H}$), 2.15 (3H, s, COC$\underline{H}_3$)

IR v_{max} (Nujol): 3470, 3410, 2500-3400, 1705, 1670 and 1615cm^{-1}

Found: C 54.49 H 4.34 N 6.38

C$_9$H$_3$NFO$_3$

requires: C 54.83 H 4.09 N 7.10%

(c) 4-Fluoroanthranilic acid

2-(Methylcarbonylamino)-4-fluorobenzoic acid (Example 9(b)) (0.45g 2.3mmol) was suspended in water (15ml) and 5M sodium hydroxide solution was added (1.8ml, 9mmol).

The solution was heated at reflux for 24h and then allowed to cool. The basic solution was extracted with dichloromethane, which was discarded, and acidified. The acidic solution was extracted with ethyl acetate (2 x 25ml), and this was dried, filtered and evaporated to leave the title compound (0.36g, 100%). m.p: 193-5°C from ethanol and water

^{1}H nmr (CDCl$_3$ /d^{6}DMSO) δ: 8.0 (4H, m on br hump, aryl-$\underline{H}$ + 2 x NH and CO$_2$H), 6.5 (2H, m, aryl-$\underline{H}$)

IR ν$_{max}$ (Nujol): 3500, 3380, 2500-3400, 1665, 1600, 1570, 1490, 1225, 1180, 1145, 980, 890, 835, 760 and 620cm^{-1}

Found: C 54.19 H 3.94 N 8.83

C$_7$N$_6$NO$_2$F

requires: C 54.20 H 3.90 N 9.03%.

(d) 4'-Amidinophenyl 2-amino-4-fluorobenzoate

2-Amino-4-fluorobenzoic acid (Example 9(c)) (320mg) was dissolved in pyridine (2ml) and 4-amidinophenol (364mg) was added. Dicyclohexylcarbodiimide (425mg) was added and the mixture stirred at room temperature under nitrogen for 18h. A mixture of product and dicyclohexylurea was precipitated. This was triturated with ethanol (10ml) and filtered. The product was precipitated by addition of diethylether to the alcoholic solution. The title compound (133mg, 21%) was isolated as flaky white crystals. m.p: > 250°C from ethanol and ether

^{1}H nmr (d^{6}DMSO) δ: 9.5 (4H, br s, amidine-N$\underline{H}$), 8.0 (3H, m, amidine aryl-$\underline{H}$ + benzoate aryl-$\underline{H}$), 7.5 (2H, d, J = 8Hz, amidine aryl-$\underline{H}$), 7.0 (2H, s, aryl-N$\underline{H}_2$), 6.6 (1H, m, aryl-$\underline{H}$), and 6.2 (1H, m, aryl-$\underline{H}$)

IR ν$_{max}$ (Nujol): 3470, 3370, 3330, 2500-3500, 1700, 1680, 1630, 1580, 245, 1210, 1180, 1040, 745, 700cm^{-1}

Found: C 53.76 H 4.23 N 13.35

C$_{14}$H$_{13}$N$_3$O$_2$FCl. $\frac{1}{4}$ H$_2$O

requires: C 53.51 H 4.33 N 13.37%.

Example 10

4'-Amidinophenyl 2-amino-4-chlorobenzoate

Recrystallized commercial 2-amino-4-chlorobenzoic acid (345mg) and 4-amidinophenol (345mg) were dissolved in pyridine (5ml). Dicyclohexylcarbodiimide (824mg, 2eq) was added and the mixture stirred at room temperature under an atmosphere of dry nitrogen for 3d. In this time a solid was formed. This was isolated by filtration and was found to be a mixture of the title compound and dicyclohexylurea. The material was triturated with chloroform (6 x 10ml) to remove the urea. This left the title compound (81mg, 12%). m.p: 230-2°C

^{1}H nmr (d^{6}DMSO) δ: 9.20 (4H,s, aryl-amidine-N$\underline{H}$), 7.90 (3H, m, aryl-$\underline{H}$), 7.52 (2H, d, J = 9Hz, aryl-$\underline{H}$), 6.95 (3H, m, aryl-$\underline{H}$ +NH$_2$), 6.65 (1H, m, aryl-$\underline{H}$)

IR ν$_{max}$ (Nujol): 3460, 3160, 1710, 1680, 1610, 1220, 1180, 1040 cm^{-1}.

Found: C 51.79 H 4.19 N 12.57

C$_{14}$H$_{13}$N$_3$Cl$_2$O$_2$

requires: C 51.55 H 4.02 N 12.88

Example 11

4'-Amidinophenyl 2-amino-4-bromobenzoate

(a) 2-Amino-4-bromotoluene

4-Bromo-2-nitrotoluene (13.5g, 62.5mmol) and granulated tin (11.25g, 95mmol) were placed in a round bottomed flask to which concentrated hydrochloric acid (25ml) was added in small (ca 5ml) aliquots. A gentle reaction was evident during this addition period. When all the acid had been added the reaction mixture was heated and stirred at about 100°C for 4h. The solution was allowed to cool and a solution of sodium hydroxide (18.75g) in water (32ml) was added. The solution was heated at 100°C for 1h. After cooling, water (200ml) was added and the solution was filtered. The aqueous filtrate was extracted with diethyl ether (200ml). The filtered salts were also extracted with diethyl ether (200ml). The combined organic layers were dried, filtered and evaporated to leave an oil. This was purified by distillation (112°C at 4mm Hg) to leave the title compound (6.76g, 58%). ^{1}H nmr (CDCl$_3$) δ: 6.8 (3H, m, aryl-$\underline{H}$), 3.6 (2H, s, N$\underline{H}_2$), and 2.1 (3H,s,C$\underline{H}_3$).

29

(b) 2-Methyl-5-bromoacetanilide

This was synthesized in the same manner as the fluoro-analogue (Example 9(a)) in 96% yield from 2-amino-4-bromotoluene (Example 11(a)). The preparation did not require any column chromatography. m.p:
5 108-110°C from dichloromethane and 40-60° petroleum ether,
^{1}H nmr (CDCl$_3$) δ: 7.8 (2H, m, aryl-H + N-H), 7.1 (2H, m, aryl-H), 2.15 (6H, s, CH$_3$ and COCH$_3$)
IR v_{max}(Nujol): 3250, 1670, 1650, 1580, 1530, 1400, 1380, 1290, 1230, 1190, 1130, 860, 800, and 700cm^{-1}
Found: C 48.00 H 4.43 N 6.16
C$_9$H$_{10}$NOBr
10 requires: C 47.39 H 4.42 N 6.14%.

(c) 2-(Methylcarbonylamino)-4-bromobenzoic acid

This material was produced in 10mmol scale from 2-methyl-5-bromoacetanilide (Example 11(b))in the same
15 way as the fluoro-analogue (Example 9(b)). A yield of 58% was obtained. m.p: 212-5°C from methanol and water
^{1}H nmr (CDCl$_3$/d^{6}DMSO) δ: 11.4 (2H,br s, NH and CO$_2$H), 9.3 (1H, m, aryl-H), 8.2 (1H, m, aryl-H), 7.4 (1H, m, aryl-H), 2.3 (3H, s, aryl-CH$_3$)
IR v_{max} (Nujol): 3250, 1670, 1650, 1580, 1520, 1290, 860 and 800cm^{-1}
20 Found: C 41.43 H 3.08 N 5.31
C$_9$H$_8$BrNO$_3$
requires: C 41.89 H 3.12 N 5.43%.

(d) 4-Bromoanthranilic acid

25
The title compound was prepared in 48% yield from 2-(methylcarbonylamino)-4-bromobenzoic acid (Example 11(c)) in the same manner as the fluoro-analogue (Example 9(c)). ^{1}H nmr (CDCl$_3$/d^{6}DMSO) δ: 8.1 (3H, s, CO$_2$H + NH$_2$), 7.6 (1H, d, J=8Hz, aryl-H), 6.8 (1H, m, aryl-H), 6.6 (1H, m, aryl-H).

30 ## (e) 4'-Amidinophenyl 2-amino-4-bromobenzoate

2-Amino-4-bromobenzoic acid (Example 11(d)) (0.90g) and 4-amidinophenol (0.718g) were dissolved in pyridine (10ml). Dicyclohexylcarbodiimide (1.71g, 2eq) was added. The reaction was stirred at room temperature under an atmosphere of dry nitrogen for 2d. It was then filtered and the solid obtained was found
35 to be a mixture of the title compound and dicyclohexylurea. The material was repeatedly triturated with chloroform (6 x 20ml) before being isolated by filtration and evaporation. The pure title compound was obtained (144mg, 9%). ^{1}H nmr (d^{6}DMSO) δ: 9.20 (4H, 6r s, amidine-NH), 7.90 (3H, m, aryl-H), 7.55 (2H, d, J=6Hz, aryl-H), 7.1 (1H, s, aryl-H), 6.95 (2H, br s, NH$_2$), 6.80 (1H, m, aryl-H)
IR v_{max} (Nujol): 3450, 3200, 1710, 1680, 1610, 1230, 1180, 1040cm^{-1}.

40
Example 12

General methods for the preparation of active-centre acylated derivatives of tissue plasminogen activator and
45 urokinase

Two-chain t-PA (prepared as in Browne, et al., Thrombosis and Haemostasis 54, 422-424 (1985)) and UK (Serono, Freiburg, W.Germany) stock were dissolved in a 10:1 mixture of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v Tween 80[3] pH 7.4 : 0.25M lysine, 100mg/ml D-mannitol, 10mM 6-aminohexanoic
50 acid pH 7.4 (1.1ml) to give final concentrations of enzyme between 50,000 and 100,000 SU ml^{-1}. This material was treated with a 6-10mM solution of acylating agent in dimethylsulphoxide. The acylations were generally conducted at 0°C overnight, although on occasion, the temperature was raised to 25°C for up to 1h to permit greater than 90% acylation to take place as judged by chromogenic substrate assay. The acyl-enzyme was desalted into 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v Tween 80, pH 7.4 (PST Buffer) by
55 passage through a pre-packed PD10 column (Sephadex G25M, Pharmacia). The acyl-enzyme was collected into PST buffer (2ml). Several aliquots (0.1ml) of this stock solution were diluted into prewarmed (37°C) buffer (0.9ml) and the rate of deacylation was determined by the return of amidolytic activity as described in the methods section and with the results given in Table 7.

60

65 [3] Tween 80 is a tradename for a detergent comprising polyoxythylene sorbitan monooleate having a molecular weight of approximately 1300 and containing approximately 18 to 22 oxyethylene units.

Table 7

Acylating Agent	Acyl-Urokinase		Acyl-Tissue Plasminogen Activator	
	Number of equivalents of acylating agent used in preparation	Half life for deacylation min. (No of determinations)	Number of equivalents of acylating agent used in preparation	Half life for deacylation min. (No of determinations)
4'-Amidinophenyl 2-amino-4-fluorobenzoate	3	97 (2)	–	–
4'-Amidinophenyl 2-amino-4-chlorobenzoate	3	59 (4)	3	110 (4)
4'-Amidinophenyl 2-amino-4-bromobenzoate	12	34 (4)	12	72 (4)

Example 13

2-Amino-4-chlorobenzoyl derivative of a conjugate of urokinase with the active centre of human plasmin

A solution of the conjugate between iminothiolane-treated human urokinase and the acyl-enzyme 4-[N-2-(3-[2-pyridyldithio]propionyl)aminoethylamino]benzoyl human plasmin (Example 10 of EP-A-0155388, 0.25ml in 0.5M 1-arginine, 0.02M trishydroxymethyaminomethane, 0.5M sodium chloride, 0.01% w/v Tween 80 detergent pH 7.4) was diluted to 1.0ml with 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% w/v Tween 80 pH 7.4 (phosphate buffer). This solution was assayed against substrate S-2444 and contained 31500 SU/ml. A solution of the acylating agent of Example 10 (20µl of 10mM in dimethylsulphoxide) was added and the mixture held at 0°C for 2h after which time the activity had declined to 12440 SU/ml. A further 20µl of acylating agent was added and incubation continued at 25°C for 30 min, the product being then held on ice. The final activity was 1875 SU/ml (6%). The mixture was gel filtered on a small column of Sephadex G-25M (PD10 column, Pharmacia) into phosphate buffer containing 1.0mM 6-aminohexanoic acid. The product was stored in solution at -70°C. Deacylation of the product, diluted 1:10 v/v in phosphate buffer at 37°C, indicated that the acyl-enzyme deacylated with an average first order rate constant of $2.64 \pm 0.09 \times 10^{-4}$ sec^{-1} half life : 43.8 min).

Example 14

2-Amino-4-fluorobenzoyl derivative of a conjugate of urokinase with the active centre of human plasmin

The urokinase-plasmin conjugate of Example 13 above (209900 SU/ml, 2.1ml in the phosphate buffer referred to above but containing 5.0mM 6-aminohexanoic acid) was treated with a solution of the acylating agent of Example 9 above (41µl of 50mM in dry dimethylsulphoxide) and incubated at 25°C. After 45 min, a further 10.5µl of 50mM acylating agent were added to bring the final concentration to 1.25 mM. After a further 40 min at 25°C, the amidolytic activity of the mixture had declined to 3.5% of the initial activity and the product was gel filtered on a small column of Sephadex G25M into the above phosphate/6-aminohexanoic acid buffer (3.4ml). The product was stored in frozen solution at -196°C. Deacylation at 37°C in phosphate buffer gave a mean first order deacylation rate constant of 1.0×10^{-4} sec^{-1}. Two other batches of this material, prepared in a similar fashion, gave acyl-enzymes with deacylation rate constants of 0.94 and 1.43×10^{-4} sec^{-1} average half life : 101.6 min).

Example 15

2-Amino-4-chlorobenzoyl des (cys$_{52}$-asp$_{87}$) rt-PA

Des (cys$_{51}$-asp$_{87}$) rt-PA (Example 4B of EP-A-O 207 589) was produced from mouse C127 cells in a two chain form and was purified essentially as described for native rt-PA (Dodd et al Febs Lett., 1986, 209, 13 - 17). Purified t-PA mutein (1.5ml; 5,300 SU/ml; approx. 1.5nmol) in 0.05M phosphate/0.1M NaCl/0.01% Tween 80/10mg ml^{-1} mannitol/1mM EACA pH 7.4 (PST/ME) was mixed with 4'- amidinophenyl 2-amino-4-chloroben-zoate (Example 10 1.5µl; 10mM in DMSO). After 30 min at 25°C the activity of the solution was 2100 SU/ml, equivalent to 60% inhibition. Further gradual additions of acylating agent was made such that the final acylating agent concentration was 60µM. At an overall incubation period of 135 min, the residual activity in the solution was 410 SU/ml, equivalent to 92% inhibition. Excess acylation agent was removed by buffer-exchange into PST/ME (2.6ml) using Sephadex G25. The material eluting in the void volume (2-amino-4-chlorobenzoyl t-PA mutein) was stored at -70°C.
A sample of the product was later deacylated by diluting it with 2ml PST, incubating at 37°C and monitoring the amidolytic activity as described in the Methods Section. 78% of the original amidolytic activity returned. In subsequent batches the mean deacylation rate constant was found to be 7.8×10^{-5} sec^{-1} (half life: 149 ± 8 min, 70 determinations).

Example 16

2-Amino-4-chlorobenzoyl (lys$_{78}$n plasmin A chain) ile$_{276}$ t-PA

Purified lys$_{78}$ plasmin A chain/ile$_{276}$ t-PA hybrid plasminogen activator synthesized essentially as described in Examples 1 and 2 of EP-A-0 155 387 (9.2ml; 66,000 SU/ml, approx. 40nmol) in PST/ME Buffer (Example 15) was mixed with 4'-amidinophenyl 2-amino-4-chlorobenzoate (Example 10) (50µl; 10mM in DMSO). After 60 min incubation at 25°C the residual amidolytic activity of the preparation was 1900 SU/MI, equivalent to 3% of the original activity. Excess acylating agent was removed by buffer-exchange into PST/ME using a column

(245 x 26mm) of Sephadex G25. The acylated hybrid plasminogen activator eluted at the void volume of the column and it was stored at -70°C.

A sample of the acylated hybrid plasminogen activator was subsequently diluted with 19 vol. PST and incubated at 37°C. The amidolytic activity of the preparation was monitored as described in in the Methods Section. The results indicated that 96% of the hybrid was in the acylated form prior to deacylation. In subsequent batches the deacylation rate constant was found to be 8.9×10^{-5} sec^{-1} (half life : 130 $\pm$ 5 min, mean of 11 determinations).

Example 17

The 2-amino 4-fluorobenzoyl derivative of a conjugate of low molecular weight urokinase with the active centre of human plasmin

A conjugate was prepared between thiolated human urokinase and the acyl-enzyme 4-[N-2-(3-[2-pyridyldi-thio]-propionyl aminoethylamino]benzoyl human plasmin as described in Example 10 of EP-A-0155388 except that low molecular weight (33 kilodalton) urokinase was employed instead of the high molecular weight form (54 kilodalton). The two forms are distinguished by the absence, in the 33K molecule, of the N-terminal 135 amino-acids. The conjugate had a molecular weight of about 110 kilodaltons. This conjugate (0.8 ml in 0.5M L-arginine, 0.5M sodium chloride, 0.02M trisaydroxymethylaminomethane, 0.01% w/v tween 80 detergent pH 7.4, activity: 290,000 SU/ml against S-2444) was treated with the acylating agent of Example 9 (40 µl of 20mM in dry dimethylsulphoxide) for 30 min at 25°C. Partial (80%) acylation occurred and the product was gel-filtered into the phosphate/6-aminohexanoic acid buffer described in Example 6 above (3.4 ml) and acylation repeated using the same conditions as above. The activity decreased to 2.5% of the starting level and the product was reduced in volume to 1.5ml by centrifugal concentration (Amicon C-30, 2000g, 45 min, 4°C). After gel filtration as described above, the product (3.4 ml) was stored at -196°C. Deacylation at 37°C in phosphate buffer gave a mean first order deacylation rate constant of 1.12×10^{-4} sec^{-1} (average half-life: 103.3 min). A second set of determinations gave a value of 0.97×10^{-4} sec^{-1}. In the figures:
(N.B. Not to scale)

Fig. 1a Structure of pTRH6
▬▬▬ = pUC8 sequences
▬▬▬ = plasminogen cDNA sequences
◄▬ = restriction sites

Fig. 1b Structure of pTRH3
▬▬▬ = pTRE12 sequences
▬▬▬ = plasminogen cDNA sequences (Hind III at 5′ end, BGIII at 3′ end).
◄▭ = restriction sites used in construction

Fig 2 Structure of pTRH9

Fragments (I) to (III) and linker (IV) are indicated.
▬▬▬ = pTRE12 D Nar Sequences
▬▬▬ = Insert Sequences
PlgnA = plasminogen A chain coding region
◄▭ = restriction sites used in construction
◄▬ = other restriction sites
├──┤ = protein coding regions
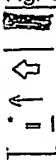
Fig. 3 Structure of pTRH20
▬▬▬ = vector sequences
▬▬▬ = insert DNA
◄▭ = restriction sites used in contruction
◄▬ = other restriction sites
* = linking amino acid sequence
├──┤ = protein coding regions

Plgn A = plasminogen A chain-coding region
t_A = t-PA A chain coding region
t_B = t-PA B chain coding region

Fig. 4 Structure of pTRH204

Fragments (V) (Sstl-Narl), (VI)(Banll-Sstl) and linker (VII)(Narl-Banll) are indicated. ▬▬▬ = vector

sequences

—— = insert DNA

◁⊐ = restriction sites used in construction

←— = other restriction sites

5 * = serine equivalent to plasminogen serine 545/t-PA serine 262 at junction

⊢—⊣ = protein coding regions

PlgnA = plasminogen A chain coding region (glu-1 to pro-544)

t-PA = t-PA coding region (thr-263 to pro-527)

10 Fig.5 Structure of pTRH37

Fragments (IX)(SstI-BstXI),(X)(BstXI-AvaII) and linker (XI) (AvaII-BanII) are indicated. ▨▩▩ = vector sequences

—— = insert DNA

15 ◁⊐ = restriction sites used in construction

←— = other restriction sites

⊢—⊣ = protein coding regions

Plgn = plasminogen coding sequences (glu_1-cys_{541})

t-PA = t-PA coding sequences (thr_{263}-pro_{527})

20 * = serine equivalent to plasminogen $serine_{545}$/t-PA $serine_{262}$ at junction

Fig. 6 Structure of pTRH34

25 Fragments (V)(SstI-NarI), (VI)(BanII-SstI) and linker (VIII)(NarI-BanII) are indicated. ▨▩▩ = vector sequences —— = insert DNA

◁⊐ = restriction sites used in construction

←— = other restriction sites

⊢—⊣ = protein coding regions

30 PlgnA = plasminogen A chain coding region (glu-1 to arg-561)

t-PA B = t-PA B chain coding region (ile-276 to pro-527)

Fig. 7 Construction of pTRH00

35 Fragment I : is a HindIII/SfaNI fragment from the lys_{78}-cassette plasmid.

Fragment II : is a SfaNI/BStXI fragment from pTRH34 containing most of K1ᵖ, all of K2ᵖ and K3ᵖ and the N terminal section of K4ᵖ.

Fragment III: is a BstXI/HindIII fragment from pTRH204 containing most of K4ᵖ, all of K5ᵖ, Bᵗ and the vector sequences from pTRE12.

40 Fig. 8 Construction of pTRH01

Fragment I : is a HindIII/BamHI fragment from pTR6F encoding the t-PA finger domain and associated N-terminal and C-terminal amino acid sequences i.e. [SYQVI] Fᵗ [HSVPVKSTRATPGS], plus the 5' untranslated 45 region and signal/pro regions.

Fragment II : is a HindIII/KpnI fragment from pTRH00 containing almost all of K1ᵖ, all of K2ᵖ, K3ᵖ, K4ᵖ, K5ᵖ, Bᵗ and the vector functions of pTRE12.

Linker: as described in Example 5.

50 Fig. 9 Construction of pTRH02

Fragment II : is a HindIII/BamHI fragment from pTR6FG encoding the t-PA Fᵗ and Gᵗ domains and associated linking N/C terminal sequences i.e. [SYQVI]Fᵗ[HSVPVKS]Gᵗ[EIDTRATPGS], plus the 5' untranslated region and signal/pro regions.

55 Fragment I : is a HindIII/KPnI fragment from pTRH00 containing almost all of K1ᵖ, all of K2ᵖ, K3ᵖ, K4ᵖ, K5ᵖ, Bᵗ and the vector functions of pTRE12.

Linker (W + X) is as described in Example 6.

Fig.10 Structure of pTRH25

60 Fragment I = BglII-NarI fragment from pTRH9.

Fragment II = SstI - BglII fragment encoding urokinase B chain

III= oligonucleotides Y + Z

▉▉ = pTRE12 sequences

65 —— = insert sequences.

←——— = restriction sites used in construction
Plgn = plasminogen coding region (glu-1 to phe-546).
u = urokinase coding region (pro-137 to leu-411).

Fig. 11

Plasmid PSV_2dhfr

P   =   Simian virus polyadenylation ⎞
          sequence   ⎪
                                         ⎬ SV_{40} 3'
I   =   Simian virus$_{40}$ intron   ⎪
          sequence   ⎠

DH = dihydrofolate reductase cDNA
$SV_{40}E$ = Simian virus$_{40}$ early promoter
EcoRI = Restriction enzyme site: EcoRI, linkered with XhoI linkers.
BglII = Restriction enzyme site: BglII.

Fig. 12 Plasmid BPV-MT-XhoI
BPV = Bovine papillomavirus sequences
MT-I = Mouse metallothionein gene sequences - including 3' polyadenylation sequence (0.3Kb fragment B)
SstI = Restriction enzyme site: SstI, linkeredwith BglII linkers.
HindIII = Restriction enzyme site: HindIII, linkered with XhoI linkers.

Fig. 13 Plasmid pTRH69
MMT 3' = Mouse metallothionein polyadenylation sequences: Fragment B excised from plasmid BPV-MT-XhoI.

DH   = dihydrofolate reductase cDNA ⎞ Fragment A
$SV_{40}E$ Simian virus$_{40}$ early promoter ⎛excised from
                                              ⎬plasmid
                                              ⎠PSV_2dhfr

XhoI = Restriction enzyme site XhoI
BglII = Restriction enzyme site BglII

Fig. 14 Plasmid pTRH71

LTR   = Rous sarcoma virus long terminal⎞
          repeat   ⎛ 3.3Kb
SV_{40}3' = Simian virus$_{40}$intron and   ⎨ fragment from
          polyadenylation sequences   ⎪ pTRE24
MUT   = t-PA Mutein cDNA   ⎠

MMT3' = Mouse metallothionein polyadenylation sequences
DH = dihydrofolate reductase cDNA
$SV_{40}E$ = Simian virus early promoter

35

Fig. 15 Plasmid pTRH11

```
LTR      = Rous sarcoma virus long terminal ⎞
           repeat                            ⎛  4.1Kb
SV₄₀3'   = Simian virus₄₀intron and          ⎛  fragment
           polyadenylation sequences         ⎝  from
H204     = H204 hybrid protein cDNA          ⎠  pTRH204
```

MMT3' = Mouse metallothionein polyadenylation sequences
DH = Dihydrofolate reductase cDNA
SV40E = Simian virus40 early promoter

Claims

1. A hybrid plasminogen activator which comprises the five kringle domains of plasminogen linked to the B-chain of t-PA or u-PA via an amino acid sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA.

2. A hybrid plasminogen activator according to claim 1 of the formula:

$(Y-)_m(K^P-Z^t-)_5B^t$

where B^t comprises residues 276-527 of t-PA, m is 1, each of the 5 values of K^P represents a kringle domain derived from plasminogen in sequence and Y and each of the 5 values of Z^t independently represents a bond or a linking sequence of amino acids which may be introduced synthetically during the preparation of the hybrid plasminogen activator and/or derived from native plasminogen and/or t-PA sequences, the sequence Z_5^t comprising at least residues cys-264 and arg-275 of t-PA.

3. A hybrid plasminogen activator according to claim 2 wherein Y represents plasminogen residues 1 to 83 or 78 to 83 respectively.

4. A hybrid plasminogen activator according to claim 2 or 3 wherein Z_1^t, Z_2^t, Z_3^t and Z_4^t represent the native plasminogen inter-domain sequences between plasminogen kringle domains 1 and 2, 2 and 3, 3 and 4 and 4 and 5, respectively.

5. A hybrid plasminogen activator according to any of claims 2 to 4 wherein Z_5^t is selected from:

```
1.     [AAPSTCGLRQYSQPQFR]

2.     [AAPSTCGLRQYSQPQFQ]

3.       [STCGLRQYSQPQFR]
```

(single letter amino acid notation).

6. Plasminogen 1-544/t-PA 262-527 including one and two chain variants, lys78 and glu1 variants, and mixtures thereof.

plasminogen 1-544/t-PA 262-527 (arg275 -> gln) including one and two chain variants, lys78 and glu1 variants, and mixtures thereof,

plasminogen 1-541/t-PA 262-527 including one and two chain variants, lys78 and glu1 variants, and mixtures thereof,

t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly-3, ser1 and val4 variants, and mixtures thereof,

t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly-3, ser1 and val4 variants, and mixtures thereof or

plasminogen 1-546/u-PA 137-411 including one and two chain variants, lys78 and glu1 variants, and mixtures thereof.

7. A hybrid plasminogen activator according to any preceding claims expressed using chinese hamster ovary cells.

8. A hybrid plasminogen activator according to any preceding claim wherein the catalytic site essential for fibrinolytic activity is blocked by a removable blocking group.

9. A hybrid plasminogen activator according to claim 8 wherein the removable blocking group is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electon-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} s c^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C.

10. A hybrid plasminogen activator according to claim 9 wherein the removable blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

11. 2-Amino-4-chlorobenzoyl plasminogen 1-544/t-PA 262-527 (two chain), including glu_1 and lys_{78} variants and mixtures thereof,
2-amino-4-chlorobenzoyl plasminogen 1-541/t-PA 262-527 (two chain), including glu_1 and lys_{78} variants and mixtures thereof or
2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (two chain), including gly_{-3}, ser_1 and val_4 variants and mixtures thereof.

12. A pharmaceutical composition comprising a hybrid plasminogen activator according to any preceding claim in combination with a pharmaceutically acceptable carrier.

13. A hybrid plasminogen activator according to any of claims I to 11 for use as an active therapeutic substance.

14. A hybrid plasminogen activator according to any of claims I to 11 for use in the treatment of thrombotic diseases.

15. Use of a hybrid plasminogen activator according to any of claims 1 to 11 in the preparation of a medicament for the treatment of thrombotic diseases.

16. A process for preparing a hybrid plasminogen activator according to any of claims 1 to 7 which process comprises expressing DNA encoding said hybrid plasminogen activator in a recombinant host cell and recovering the hybrid plasminogen activator product.

17. A DNA polymer comprising a nucleotide sequence that encodes a hybrid plasminogen activator according to any of claims 1 to 7.

18. A derivative of a plasminogen activator comprising the serine protease domain of t-PA or u-PA, in which the catalytic site essential for plasminogen activator activity is blocked by a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electon-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C.

19. A derivative according to claim 18 wherein the pseudo first order rate constant is in the range 7.0×10^{-5} to 1.5×10^{-4} s^{-1}.

20. A derivative according to claim 18 or 19 wherein the blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

21. A derivative according to claim 18 selected from
4-fluoro-2-aminobenzoyl urokinase,
4-chloro-2-aminobenzoyl urokinase or t-PA, and
4-bromo-2-aminobenzoyl t-PA;
2-amino-4-chlorobenzoyl(lys_{78} plasmin A-chain)ile$_{276}$t-PA;
the 4-chloro- and 4-fluoro-2-aminobenzoyl derivatives of the conjugates in which urokinase (high or low molecular weight) is linked reversibly to the active centre of human plasmin; and
the 4-chloro-2-aminobenzoyl derivative of des(cys$_{51}$-asp$_{87}$)t-PA.

22. A pharmaceutical composition comprising a derivative according to any of claims 18 to 21 in combination with a pharmaceutically acceptable carrier.

23. A derivative according to any of claims 18 to 21 for use as an active therapeutic substance.

24. A derivative according to any of claims 18 to 21 for the treatment of thromboembolic diseases.

25. Use of a derivative according to any of claims 18 to 21 for the manufacture of a medicament for the treatment of thromboembolic disease.

26. A process for preparing a derivative according to any of claims 18 to 21 which process comprises reacting the plasminogen activator with a blocking agent JK in which J is a locating group which mediates binding of the agent to the catalytic site of the enzyme and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups.

27. A blocking agent of the formula JK in which J is a locating group which mediates binding of the agent to the catalytic site of a serine protease and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups.

28. 4-Amidinophenyl 2'-amino-4'-fluorobenzoate, 4-amidinophenyl 2'-amino-4'-chlorobenzoate or
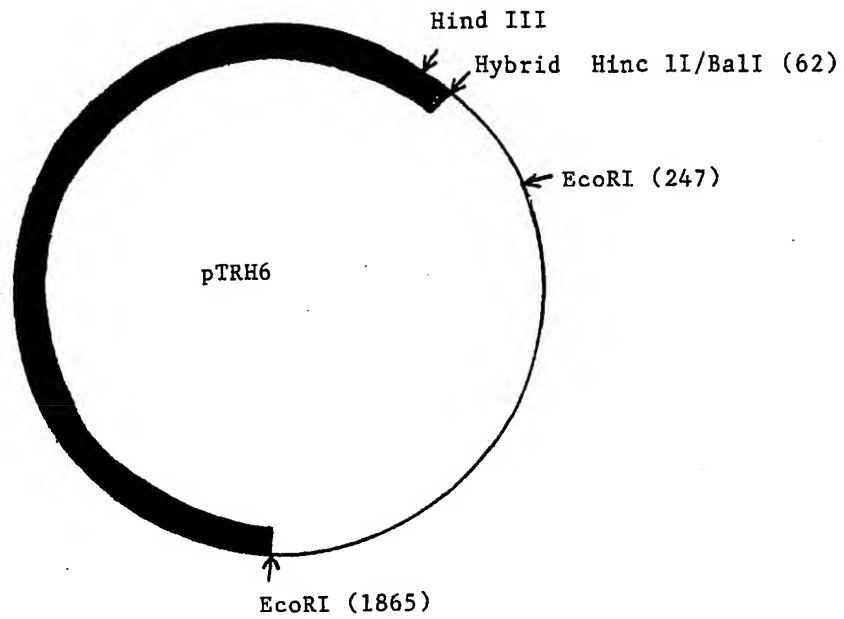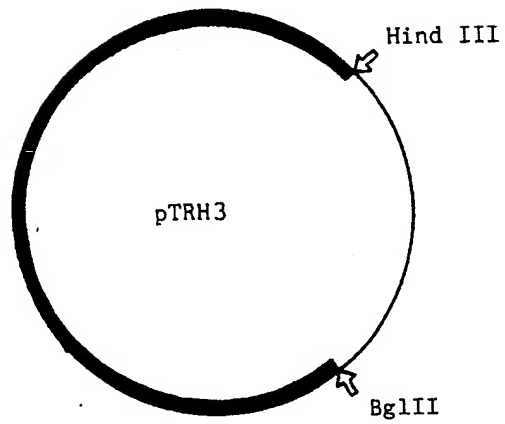
4-amidinophenyl 2'-amino-4-bromobenzoate.

Claims for the f ll wing Contracting Stat : ES
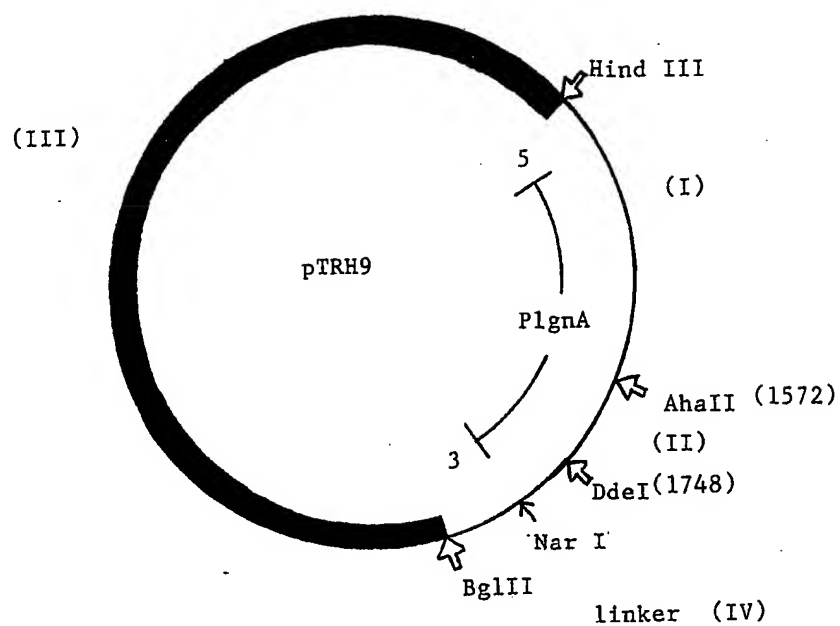
5   1. A process for preparing a hybrid plasminogen activator which comprises the five kringle domains of plasminogen linked to the B-chain of t-PA or u-PA via an amino acid sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA and wherein the catalytic site essential for fibrinolytic activity is optionally blocked by a removable blocking group, which
10   process comprises expressing DNA encoding said hybrid plasminogen activator in a recombinant host cell and recovering the hybrid plasminogen activator product, and thereafter optionally blocking the catalytic site essential for fibrinolytic activity with a removable blocking group.
   2. A process according to claim 1 for preparing a hybrid plasminogen activator of the formula:
   $(Y-)_m(K^p-Z^t-)_5B^t$

15   where B^t comprises residues 276-527 of t-PA, m is 1, each of the 5 values of K^p represents a kringle domain derived from plasminogen in sequence and Y and each of the 5 values of Z^t independently represents a bond or a linking sequence of amino. acids which may be introduced synthetically during the preparation of the hybrid plasminogen activator and/or derived from native plasminogen and/or t-PA sequences, the sequence Z_5^t comprising at least residues cys-264 and arg-275 of t-PA.

20   3. A process according to claim 2 wherein Y represents plasminogen residues 1 to 83 or 78 to 83 respectively.
   4. A process according to claim 2 or 3 wherein Z_1^t, Z_2^t, Z_3^t and Z_4^t represent the native plasminogen inter-domain sequences between plasminogen kringle domains 1 and 2, 2 and 3, 3 and 4 and 4 and 5, respectively.

25   5. A process according to any of claims 2 to 4 wherein Z_5^t is selected from:

   1.   [AAPSTCGLRQYSQPQFR]

30   2.   [AAPSTCGLRQYSQPQFQ]

   3.   [STCGLRQYSQPQFR]

   (single letter amino acid notation).
35   6. A process according to claim 1 for preparing plasminogen 1-544/t-PA 262-527 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof,
   plasminogen 1-544/t-PA 262-527 (arg_{275} -> gln) including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof,
   Plasminogen 1-541/t-PA 262-527 including one and two chain variants, lys_{78} and glu_1 variants, and
40   mixtures thereof,
   t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly_{-3}, ser_1 and val_4 variants, and mixtures thereof,
   t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly_{-3}, ser_1 and val_4 variants, and mixtures thereof or
45   plasminogen 1-546/u-PA 137-411 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof.
   7. A process according to any preceding claim wherein the host cell is a chinese hamster ovary cell.
   8. A process for preparing a hybrid plasminogen activator according to any preceding claim, wherein the removable blocking group is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen
50   atom and optionally further substituted with one or more weakly electron-withdrawing or electon-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0 $\times 10^{-5}$ to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300. at pH 7.4 at 37°C.
55   9. A process according to claim 8 wherein the removable blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.
   10. A process according to claim 1 for preparing 2-amino-4-chlorobenzoyl plasminogen 1-544/t-PA 262-527 (two chain), including glu_1 and lys_{78} variants and mixtures thereof,
   2-amino-4-chlorobenzoyl plasminogen 1-541/t-PA 262-527 (two chain), including glu_1 and lys_{78} variants
60   and mixtures thereof, or
   2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (two chain), including gly_{-3}, ser_1 and val_4 variants and mixtures thereof.
   11. A process for preparing a pharmaceutical composition comprising a hybrid plasminogen activator as defined in any preceding claim in combination with a pharmaceutically acceptable carrier which process
65   comprises admixing said activator and said carrier.

12. Use of a hybrid plasminogen activator as defined in any of claims 1 to 10 in the preparation of a medicament for the treatment of thrombotic diseases.

13. A method of treating thrombotic diseases, which comprises administering to the sufferer an effective, non-toxic amount of a hybrid plasminogen activator as defined in any of claims 1 to 10.

14. A process for preparing a DNA polymer comprising a nucleotide sequence that encodes a hybrid plasminogen activator as defined in any of claims 1 to 6 by the condensation of appropriate mono-, di- or oligomeric units.

15. A process for preparing a derivative of a plasminogen activator comprising the serine protease domain of t-PA or u-PA, in which the catalytic site essential for plasminogen activator activity is blocked by a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electon-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C, which process comprises reacting the plasminogen activator with a blocking agent JK in which J is a locating group which mediates binding of the agent to the catalytic site of the enzyme and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups.

16. A process according to claim 15 wherein the pseudo first order rate constant is in the range 7.0×10^{-5} to 1.5×10^{-4} s^{-1}.

17. A process according to claim 15 or 16 wherein the blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

18. A process according to claim 15 for preparing a derivative selected from
4-fluoro-2-aminobenzoyl urokinase,
4-chloro-2-aminobenzoyl urokinase or t-PA, and
4-bromo-2-aminobenzoyl t-PA;
2-amino-4-chlorobenzoyl(lys$_{78}$ plasmin A-chain)Ile$_{276}$t-PA;
the 4-chloro- and 4-fluoro-2-aminobenzoyl derivatives of the conjugates in which urokinase (high or low molecular weight) is linked reversibly to the active centre of human plasmin; and
the 4-chloro-2-aminobenzoyl derivative of des(cys$_{51}$-asp$_{87}$)t-PA.

19. A process for preparing a pharmaceutical composition comprising a derivative as defined in any of claims 15 to 18 in combination with a pharmaceutically acceptable carrier, which process comprises admixing said activator and said carrier.

20. Use of a derivative according to any of claims 15 to 18 for the manufacture of a medicament for the treatment of thromboembolic disease.

21. A method of treating thromboembolic diseases which comprises administering to the sufferer an effective non-toxic amount of a derivative according to any of claims 15 to 18.

22. A process for preparing a blocking agent of the formula JK in which J is a locating group which mediates binding of the agent to the catalytic site of a serine protease and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups, by the reaction of the appropriately substituted benzoic acid with the appropriate alcohol.

23. A process according to claim 22 for preparing
4-amidinophenyl 2'-amino-4'-fluorobenzoate,
4-amidinophenyl 2'-amino-4'-chlorobenzoate or
4-amidinophenyl 2'-amino-4-bromobenzoate.

Fig. 1a

Hind III
Hybrid Hinc 1I/BalI (62)

EcoRI (247)

pTRH6

EcoRI (1865)

Fig. 1b

Hind III

pTRH3

BglII

Fig. 2

Fig. 3

Fig.4

pTRH204

(V)

Hind III

5'

Plgn A

*

3'   t-PA

NarI

BglII

BanII

(1024)

linker (VII)

(VI)

SstI/BanII(1417)

Fig. 5

Hind III

(IX)

pTRH37

Plgn

BstXI (1209)

(X)

AvaII (1693)

NarI

linker (XI)

BanII (1024)

*

t-PA

(VI)

BglII

SstI/BanII (1417)

0297882

Fig. 6

(V)

Hind III

pTRH34

5'

Plgn A

t-PAB

3'

NarI

linker VIII

BanII

(1024)

BgIII

(VI)

SstI/BanII(1417)

Fig. 7

pTRH00

Hind III

Fragment I

SfaN1

Fragment III

Fragment II

BstXI

0297882

Fig. 8

pTRH01

Hind III

Fragment I

Fragment II

BamH1

Linker

KpnI

Fig. 9

pTRH02

HindIII

FragmentII

Fragment1

BamH1

Linker

KpnI

Fig.10

pTRH25

(I)

Plgn

U

Nar I

(III)

SstI

Bg1II

(II)

Fig. 11

Plasmid PSV₂dhfr

Eco RI

P

3.4Kb
FRAGMENT
A

I

Bg1II

DH

SV₄₀ᴱ
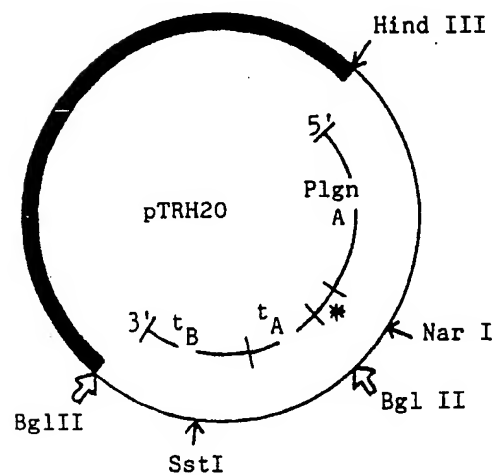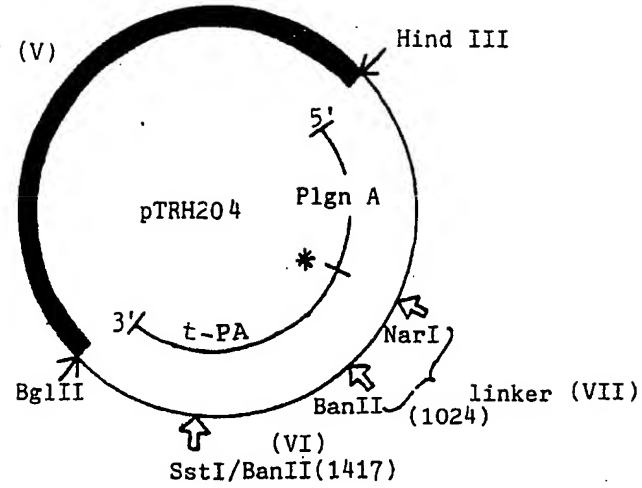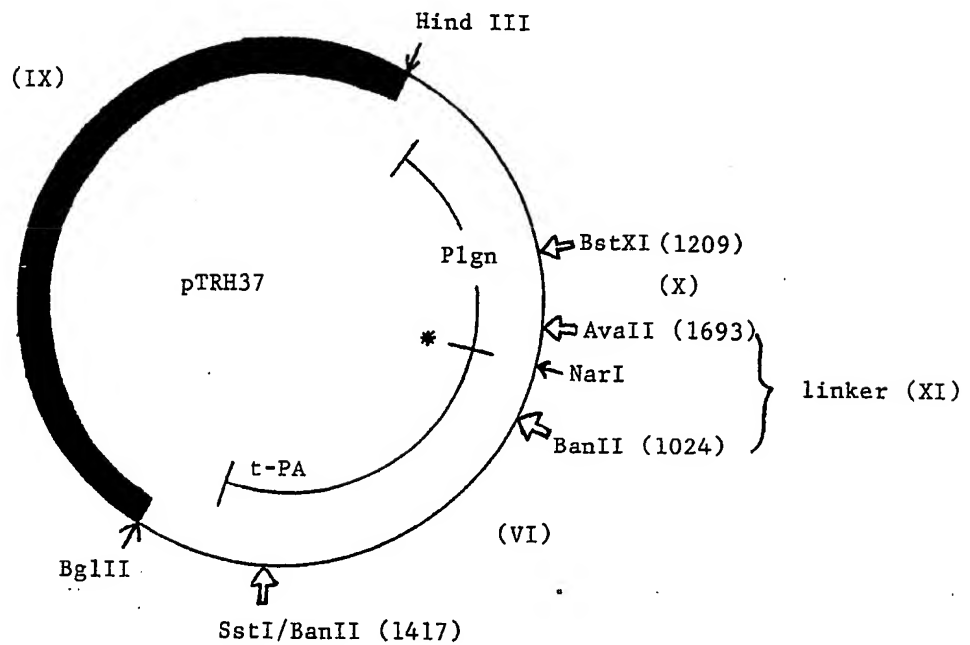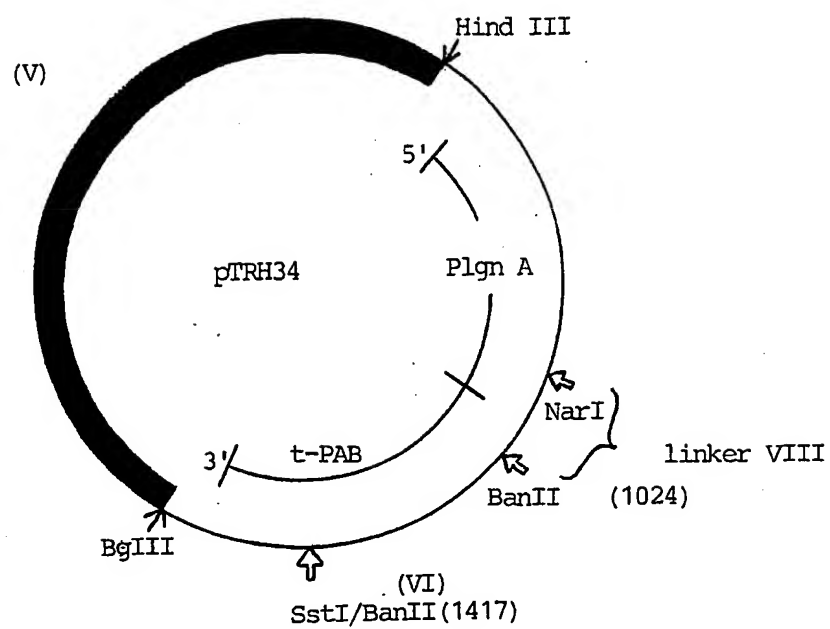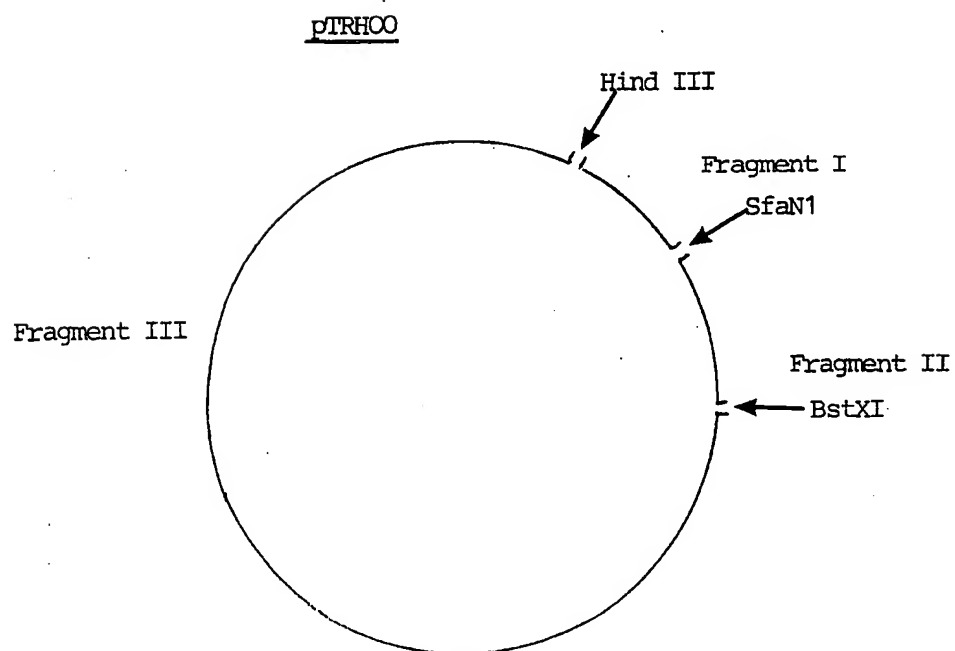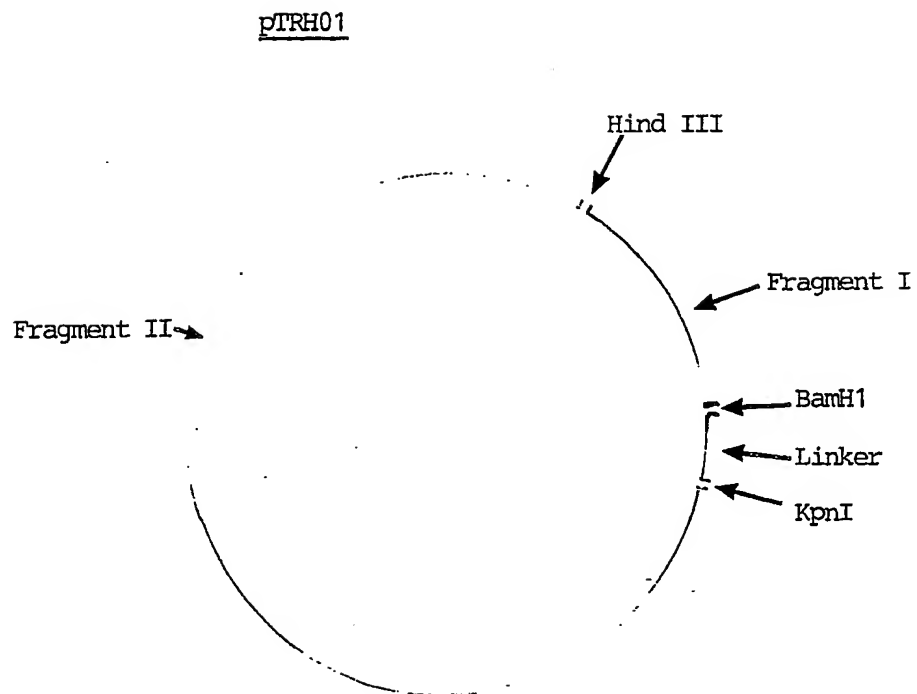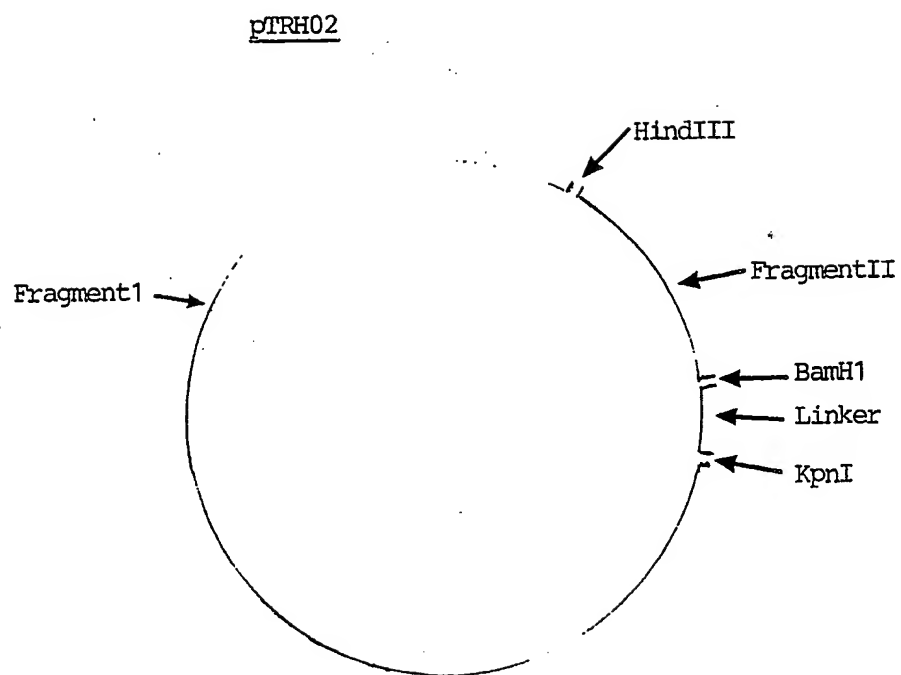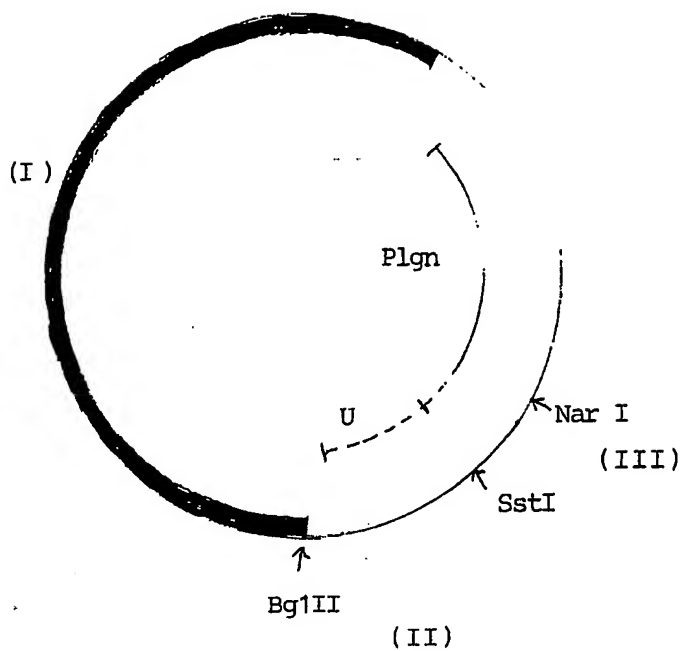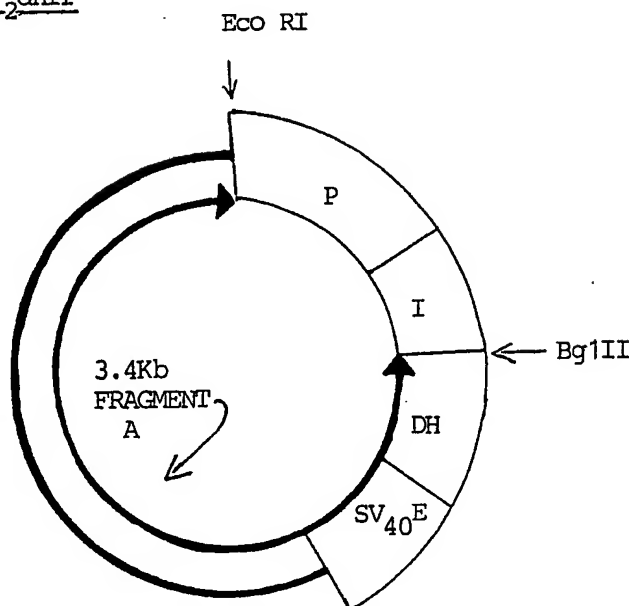
Fig. 12

Plasmid BPV-MT-XhoI
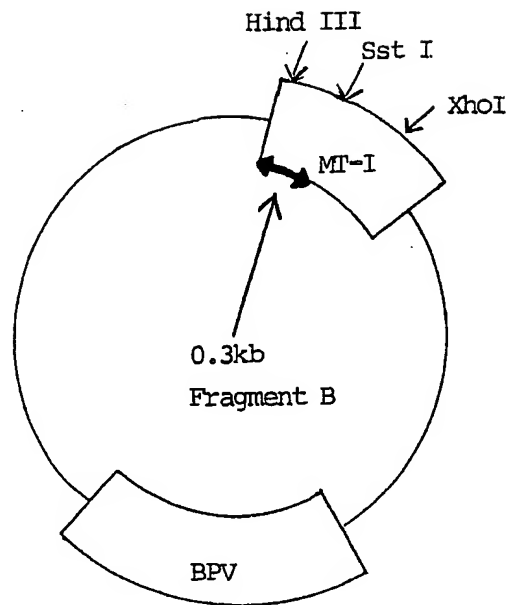
Fig.13
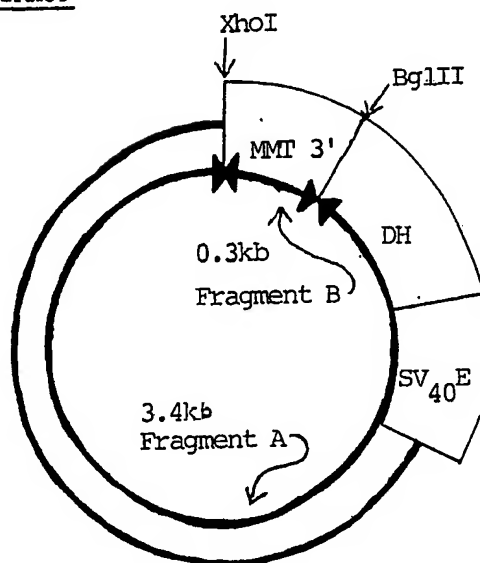
Plasmid pTRH69

Fig. 14
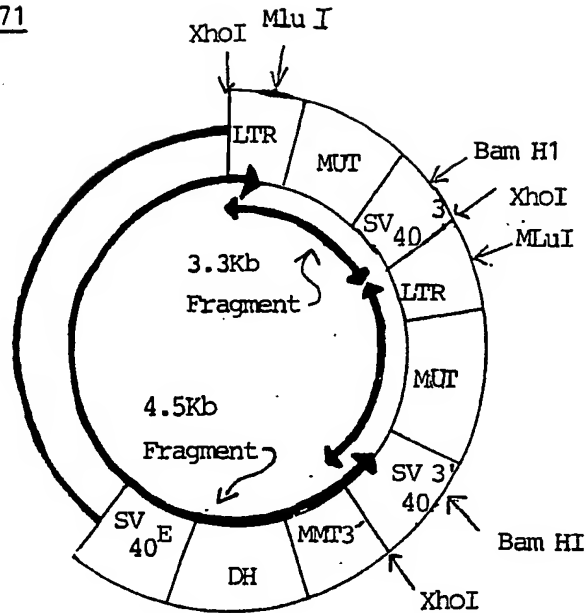
Plasmid pTRH71

Fig. 15

Plasmid pTHRH11
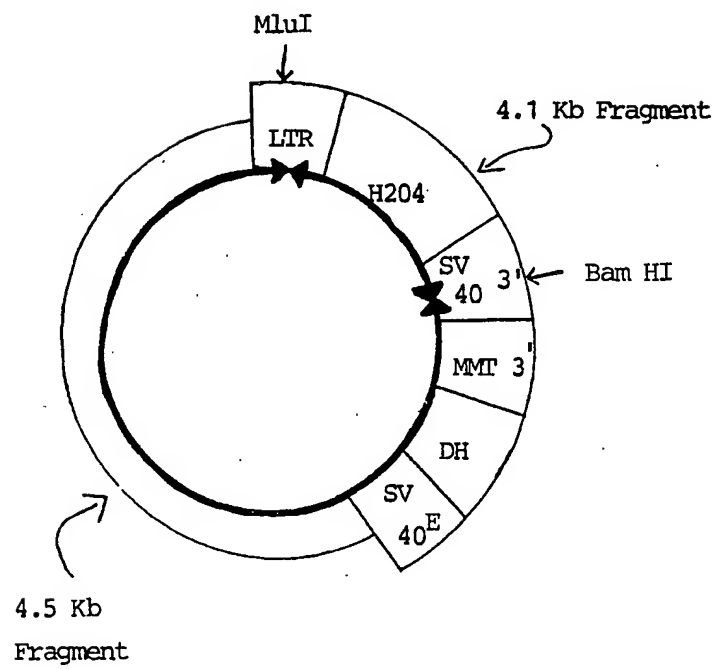
EUROPEAN PATENT SPECIFICATION

(54) Hybrid plasminogen activators.

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BIOCHEMISTRY, vol. 26, no. 15, August 1987, pp. 4661-4667; Am. Chem.Soc., Washington, US; K.C. ROBBINS et al.: "A covalent molecular wight 92000 hybrid plasminogen activator derived from human plasmin fibrin-binding and tissue plasminogen activator catalytic domains"

BIOMED. BIOCHIM. ACTA, vol. 45, no. 11/12, 1986, pp. 1405-1410; Berlin, DE; J. STÜR- ZEBECHER et al.: "Stable acyl-derivatives of trypsin-like enzymes. Preparation, kinetics, application"

THROMBOSIS RESEARCH, vol. 47, no. 6, Sep- tember 1987, pp. 699-703; Pergamon Jour- nals Ltd, New Yord, US; J. STÜRZEBECHER et al.: Stable acyl-derivatives of tissue-type plasminogen activator"

BIOLOGICAL ABSTRACTS, vol. 85, no. 6, 1988, p. AB-843, ref. no. 60983; Philadelphia, US; S. KALINDJIAN et al.: "Reagents for re- versible coupling of proteins to the active centres of trypsin-like serine proteinases"

Description

The present invention r lates to a hybrid fibrinolytic enzyme, its preparation, pharmac utical composi-
tions containing it and its use in the treatment of thrombotic disease, and to enzyme derivatives for use in
5   the treatment of thromboembolic diseases, in particular acute myocardial infarction.

European Patent No 0,009,879 discloses derivatives of in vivo fibrinolytic enzymes which are useful
therapeutic agents for treating venous thrombosis. The derivatives are characterised by the active catalytic
site on the enzymes being blocked by a group which is removable by hydrolysis such that the pseudo first
order rate constant for hydrolysis is in the range 10^{-6} to 10^{-3} sec^{-1}.

10   EP-0155387 discloses fibrinolytically active hybrid protein which comprises one chain of a 2-chain
protease linked to a chain of a different 2-chain protease, or to the same chain of the same protease, at
least one of the chains in the hybrid protein being derived from a fibrinolytically active protease, such that
the hybrid protein has a catalytic site essential for fibrinolytic activity which is optionally blocked by a
removable blocking group.

15   Examples of the hybrid protein include plasmin A-chain linked to tissue-type plasminogen activator (t-
PA) B-chain or urokinase B-chain.

The hybrid proteins may be prepared by mixing a chain of one protease with a chain of another
protease, optionally with dialysis, under oxidative conditions.

The hybrid proteins may be prepared by taking the genetic information (DNA sequence) of
20   each protein, cutting and ligating this to construct a new DNA sequence coding for the hybrid protein, and
expressing this DNA in prokaryote or eukaryote hosts.

The sequence of amino acids making up the enzyme tissue-type plasminogen activator (t-PA) and the
nucleotide sequence for the cDNA which codes for t-PA are known (see Pennica et al., 1983; Nature, 301,
214). t-PA is known to have fibrinolytic activity.

25   As used herein, the term tissue-type plasminogen activator (t-PA) denotes a plasminogen activator of
the group having the immunological properties defined for t-PA at the XXVIII Meeting of the International
Committee on Thrombosis and Haemostasis, Bergamo, Italy, 27 July 1982.

The amino acid sequence of various forms of t-PA are known. The abovementioned Nature 1983
reference discloses the sequence for the L-chain and the mature S-chain forms of t-PA, also discussed by
30   Vehar et al., Biotechnology, 1984, 2, 1051-7 in which the processing of initially formed t-PA by removal of a
pro-sequence to give the S-chain form is reported. Pohl et al., FEBS letters, 1984, Vol. 168 No.1, 29-32,
refers to the N-terminal multiplicity of t-PA and discloses the U-chain form. The numbering system for the
amino acid sequence of t-PA used herein is that described in the Nature 1983 reference for mature (S-
chain) t-PA in which the N-terminal serine is numbered 1. By this system, L-chain t-PA has an N-terminal
35   glycine residue at position -3 and U-chain t-PA has an N-terminal valine at position 4. References to t-PA
herein are understood to include all such variant forms.

Native t-PA is composed of a B or light (L) and an A or heavy (H) chain. The B-chain contains the
active site of the enzyme. The cleavage site for the conversion of t-PA from the single to the two-chain form
is located between residues arg-275 and ile-276. In the two-chain form the chains are held together by a
40   disulphide bridge formed between residues cys-264 in the A-chain and cys-395 in the B-chain.

It has been shown (Ny, T. et al, 1984; Proc. Natl. Acad. Sci. U.S.A., 81, 5355) that the A chain exhibits a
number of structural and functional domains which are homologous to structures found in other plasma
proteins: two triple disulphide-bonded structures or kringles, a growth-factor-like domain and a fibronectin-
finger-like domain.

45   The region from amino acid residues 44 to 91 has been termed the "growth factor domain" (Banyai, L.,
et al FEBS Lett. 163, 37, 1983). The genetic information which codes for the major part of this domain,
residues 51 to 86 inclusive, and partially codes for residues 50 and 87, lies on a single exon. The region
from the first to last cysteine residues within this region, residues 51 to 84 inclusive, defines a triple-
disulphide linked structure which will be referred to herein as the t-PA growth factor domain.

50   Fibronectin has twelve finger-domains per monomer, responsible for fibrin-affinity (Eur. J. Biochem. 154,
15-29 (1986)). The amino acid sequences of these finger domains are known (EMBO J.4, 1755 - 1759
(1985); Eur. J. Biochem. 128, 605-623 (1982); Proc. Natl. Acad. Sci. USA 80, 137-141 (1983)). It has been
shown (J. Biol. Chem. 260, 5328-5341 and 13666-13676 (1985)) that part of Factor XII shows structural
homology with the finger-domains of fibronectin. It has also been shown (Banyai, L. et al, 1983; FEBS Lett.,
55   163, 37)that a part of the t-PA enzyme shows structural homology with the finger-domains of fibronectin.
This region from amino acid residue 6 to 43 has been termed the t-PA finger domain. The genetic
information for this domain lies on a single exon (Ny, T. et al, 1984; Proc. Natl. Acad. Sci. U.S.A., 81, 5355).
The term "finger domain" will be used hereinafter to refer to an amino acid sequence showing structural

3

homology with the finger-domains of fibron ctin, or the sequence of a fibronectin finger-domain itself.

The s quence of amino acids making up the enzyme urokinase-type plasminogen activator (u-PA) in its single chain and two chain forms (Verstraete, M. and Collen, D., 1986; Blood, 67, 1529) and th nucleotide sequence for the cDNA which codes for human u-PA (Holmes, W. E. et al, 1985; Bio/technology 3, 923-
5   929) are known. Urokinase-type plasminogen activator is known to have fibrinolytic activity. The two chains of u-PA are termed the A- and B-chain. The B-chain contains the active site of the enzyme. The cleavage site for the conversion of u-PA from the single to the two chain form is located between residues lys-158 and ile-159. In the two chain form the chains are held together by a disulphide bridge formed between residues cys-148 in the A-chain and cys-279 in the B-chain.

10   As used herein, the term urokinase-type plasminogen activator (u-PA) denotes a plasminogen activator of the group having the immunological properties defined for u-PA at the XXVIII Meeting of the International Committee on Thrombosis and Haemostasis, Bergamo, Italy, 27 July 1982.

The numbering system for the amino acid and nucleotide sequence of u-PA used herein is that described in Holmes, W. E. et al, 1985 (op. cit.) in which the N-terminal serine residue is numbered 1.

15   In addition to the native forms of t-PA and u-PA described above, various muteins are also known, see for example EP-A-0201153, EP-A-0233013, EP-A-0199574, WO 86/01538, EP-A-0227462, EP-A-0253582, WO 86/04351, EP-A-0236040, EP-A-0200451, EP-A-0238304, EP-0225286, DE 3537176, WO 87/04722 and EP-A-0236289.

References herein to t-PA and u-PA species include both native forms and muteins.

20   Plasmin is a two-chain serine protease which may be obtained by the cleavage of the single chain precursor, plasminogen, at a specific internal peptide bond. The amino acid sequence of human plasminogen is known (Wiman and Walters (1975) Eur.J. Biochem. 50, 489-494 and 58, 539-547; Wiman (1977) Eur. J. Biochem. 76, 129-137; Sottrup-Jensen et al. (1978) Fibrinolysis and Thrombolysis Vol. 3, 191-209, Raven Press, New York; and Sottrup-Jensen et al. (1978) Atlas of Protein Sequence and Structure Vol. 5,
25   Suppl. 3, p91, National Biomedical Research Foundation, Silver Spring, MD). A partial nucleotide sequence coding for amino acid residues 272-790 of human plasminogen has also been described (Malinowski, D.P. et al., 1984, Biochemistry, 23, 4243-4250). The cleavage site of human plasminogen is located between residues arg-560 and val-561 (according to the sequence numbering of Sottrup-Jensen et al. (1978) Atlas of Protein Sequence (op.cit.)). Two species of plasminogen have been identified (F.J. Castellino, Chemical
30   Reviews Vol. 81 p431 (1981)): glu$_1$ which has an N-terminal glutamic acid residue at position 1 and lys$_{77}$ which has an N-terminal lysine residue at position 77. Glu-plasminogen is also easily converted by limited plasmic digestion to other modified forms with N-terminal valine (val$_{78}$) or methionine (met$_{68}$) (C. Miyashita, E. Wenzel and M. Heiden, Haemostasis 18, supp.1 pp 7-13 (1988)). References to plasminogen herein are understood to include all these species.

35   Recently a complete nucleotide sequence has been described (Forsgren, M., et al., 1987, FEBS Letters 213, 254-260). The nucleotide sequence predicts the existence of an extra, previously unreported, isoleucine residue near the N-terminus of the A-chain. This finding has been independently confirmed (McLean, J.N., et al., 1987, Nature 330, 132-137). Accordingly all sequence numbering (amino acid and nucleotide) below follows Forsgren et al. (1987). In this numbering sequence the plasminogen cleavage site
40   is located between residues arg-561 and val-562 and the N-terminal modified forms are termed met$_{69}$, lys$_{78}$ and val$_{79}$.

Plasminogen has five kringle structures. The region from the first to the last cysteine residue of each kringle structure, residues 84 to 162, 166 to 243, 256 to 333, 358 to 435 and 462 to 541 inclusive will be referred to herein as the K$_1$P, K$_2$P, K$_3$P, K$_4$P and K$_5$P domains respectively.

45   According to the present invention there is provided a hybrid plasminogen activator which comprises the five kringle domains of plasminogen linked to the B-chain of t-PA or u-PA via an amino acid sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA, said sequence being selected from:

50

      1.   [AAPSTCGLRQYSQPQFR]

      2.   [AAPSTCGLRQYSQPQFQ]

      3.   [STCGLRQYSQPQFR]

55

or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA, said sequence being: [AAPSFPSSPPEELKFQCGQKTLRPRFK] (single letter amino acid notation).

It will be understood that by the term 'B-chain' is meant at least that portion of the B-chain containing the functional active centre of t-PA or u-PA, and preferably comprises residues 276-527 or 159-411 respectively.

The linking sequence of amino acids may be introduced synthetically during the preparation of the hybrid plasminogen activator (PA) and/or derived from native sequences.
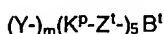
Native plasminogen includes cysteine residues at positions 548 and 558, C-terminal to plasminogen kringle 5, which participate in the interchain disulphide bridges of the two-chain plasmin form. In the preferred embodiment these residues are not present in the linking sequence.

It will be appreciated that to prevent cleavage of the plasminogen kringles from the t-PA or u-PA B-chain in vivo, the linking sequence should be chosen so as to avoid the presence of a site susceptible to trypsin-like proteolytic cleavage N-terminal to residue cys-264 of t-PA or cys-148 of u-PA, as appropriate.

Where the B-chain of t-PA is employed, the linking sequence of amino acids preferably comprises t-PA residues 264 to 275 inclusive, more preferably residues 262 to 275 inclusive.

Where the B-chain of u-PA is employed, the linking sequence of amino acids preferably comprises u-PA residues 148 to 158 inclusive, more preferably residues 137 to 158 inclusive.

In one preferred aspect, the hybrid PA may be represented symbolically as:

$$(Y-)_m (K^P - Z^t -)_5 B^t$$

where B^t comprises residues 276-527 of t-PA, m is 0 or 1, preferably 1, each of the 5 values of K^P represents a kringle domain derived from plasminogen in sequence and Y and each of the 5 values of Z^t independently represents a bond or a linking sequence of amino acids which may be introduced synthetically during the preparation of the hybrid PA and/or derived from native plasminogen and/or t-PA sequences, the sequence Z_5^t being the amino acid linking sequence above defined.

In a second preferred aspect, the hybrid PA may be represented symbolically as:
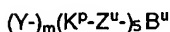
$$(Y-)_m (K^P - Z^u -)_5 B^u$$

where B^u comprises residues 159-411 of u-PA, Y and each of the 5 values of Z^u independently represents a bond or a linking sequence of amino acids which may be introduced synthetically during the preparation of the hybrid PA and/or derived from native plasminogen and/or u-PA sequences, the sequence Z_5^u being the amino acid linking sequence above defined and m and each of the 5 values of K^P are as previously defined.

When m is 1, the sequence Y may take one of the values found in various forms of native plasminogen, such as the glu_1 or lys_{78} forms, that is plasminogen residues 1 to 83 or 78 to 83 respectively.

Optionally, as taught generally in EP-A-0241210, the sequence Y may commence with one or more finger domains, defined herein as an amino acid sequence showing structural homology with the finger-domains of fibronectin, such as a fibronectin finger domain itself or a t-PA finger domain, preferably a t-PA finger domain.

Where a finger domain is derived from t-PA, the finger domain sequence may optionally extend at the N-terminus to include residues preceding residue 6 of native t-PA, such as residues 4 and 5, 1 to 5 or -3 to 5 respectively of the U-, S- or L-chain forms of native t-PA. The finger domain sequence preferably extends at the N-terminus to comprise residues 1 to 5 of native t-PA.

Each finger domain sequence may optionally be linked to a second sequence of amino acids which corresponds to the growth-factor domain of native t-PA. Thus, representing the finger domain sequence (for example residues 6 to 43 of t-PA) as F and the growth-factor domain sequence (residues 51 to 84 of t-PA) as G, Y comprises one or more N-terminal units of the form $A-F-X_1-$ and/or $A-F-X_2-G-X_3-$, where X_1, X_2 and X_3 represent bonds or linking sequences of amino acids which may be introduced synthetically during the preparation of the hybrid PA and/or be derived from native t-PA sequences adjacent the F and G domains and A is an optional N-terminal extension of the F domain.

In a preferred aspect, the linking sequence X_1 comprises amino acid residues selected from the residues 44 to 50 and 85 to 91, and more preferably comprises residues 44 to 50 and 88 to 91, of native t-PA, optionally linked at the C-terminus to a sequence of amino acids, such as -pro-gly-. The linking sequence X_2 preferably comprises residues selected from the residues 44 to 50 of native t-PA, and more preferably comprises residues 44 to 50. The linking sequence X_3 preferably comprises residues selected from the residues 85 to 91 of native t-PA and more preferably comprises residues 85 to 91, optionally linked at the C-terminus to a sequence of amino acids such as -pro-gly-.

It will be appreciated that to prevent cleavage of the additional domain(s) from one another or from the remainder of the hybrid plasminogen activator in vivo, inter-domain linking sequences and linking se-

quenc s between the additional domain(s) and the plasminogen activator molecule should be chosen so as to avoid the presence of a site susceptible to proteolytic cleavage. Thus, in particular, the linking s quence X_1 or X_3 will preferably nd with a residue other than arginine or lysine.

Y preferably comprises up to 6 additional finger domains, more preferably up to 2, most preferably 1, each of which may optionally be linked to a growth factor domain.

Z_1, Z_2, Z_3 and Z_4 preferably represent the native plasminogen inter-domain sequences between plasminogen kringle domains 1 and 2, 2 and 3, 3 and 4 and 4 and 5, respectively.

Suitable sequences (Z_5^1) linking the C-terminal plasminogen kringle domain to the t-PA B-chain are

1.   [AAPSTCGLRQYSQPQFR]

2.   [AAPSTCGLRQYSQPQFQ]

3.   [STCGLRQYSQPQFR]

(single letter amino acid notation) from which it can be seen that the sequences 1 and 2 consist of residues 542-544 of plasminogen and residues 263 to 275 of t-PA linked by a serine residue. The interposed serine residue can be identified with ser-545 of plasminogen or ser-262 of t-PA. In sequence 2, residue 275 of t-PA has been replaced by glutamine in accordance with EP-A-0233013. Sequence 3 consists of residues 262 to 275 of t-PA.

The (Z_5^u) linking the C-terminal plasminogen kringle domain to the u-PA B-chain is:
[AAPSFPSSPPEELKFQCGQKTLRPRFK]
(single letter amino acid notation) from which it can be seen that the sequence consists of residues 542-546 of plasminogen and residues 137 to 158 of u-PA.

The preferred hybrid PA's of the invention have the following structures:

1. Plg 1-541[AAPSTCGLRQYSQPQFR]B^t

2. Plg 1-541[AAPSTCGLRQYSQPQFQ]B^t

3. Plg 1-541   [STCGLRQYSQPQFR]B^t

where Plg 1-541 represents residues 1-541 of plasminogen (that is, including kringles 1 to 5), B^t is as previously defined and the symbols in brackets represent amino acid residues according to the single letter amino acid notation, including one and two chain variants, lys$_{78}$ and glu$_1$ variants, and mixtures thereof.

The hybrid PA of the invention may be derivatised to provide pharmaceutically useful conjugates analogous to known PA-containing conjugates, for example:

(a) an enzyme-protein conjugate as disclosed in EP-A-O 155 388, in which the catalytic site on the enzyme which is responsible for fibrinolytic activity is blocked by a human protein attached thereto by way of a reversible linking group;

(b) an enzyme-protein conjugate as disclosed in EP-A-O 152 736, comprising at least one optionally blocked fibrinolytic enzyme linked by way of a site other than the catalytic site responsible for fibrinolytic activity to at least one human protein;

(c) a protein-polymer conjugate as disclosed in EP-A-0183503 comprising a pharmaceutically useful protein linked to at least one water soluble polymer by means of a reversible linking group; or

(d) an enzyme conjugate as disclosed in EP-A-0184363 comprising a plurality of fibrinolytic enzymes linked together through the active centres thereof by means of a removable blocking group.

The hybrid PA of the invention may take the place of a PA as the enzyme or (human) protein component, as appropriate, of any of the conjugates described above.

The above mentioned derivatives of the hybrid PA may be used in any of the methods and compositions described hereinafter for the hybrid PA itself.

In a further aspect, the invention provides a process for preparing hybrid plasminogen activator according to the invention which process comprises expressing DNA encoding said hybrid plasminogen activator in a recombinant host cell and recovering the hybrid plasminogen activator product.

The DNA polymer comprising a nucleotide sequence that encodes the hybrid PA also forms part of the invention.

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis et. al., Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982 and DNA

Cloning vols I, II and III (D.M. Glover ed., IRL Press Ltd).

In particular, the process may comprise the steps of:

i) preparing a r plicabl expr ssion vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes said hybrid plasminogen activator;

5   ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said hybrid plasminogen activator; and

iv) recovering said hybrid plasminogen activator.

The invention also provides a process for preparing the DNA polymer by the condensation of
10   appropriate mono-, di- or oligomeric nucleotide units.

The preparation may be carried out chemically, enzymatically, or by a combination of the two methods, in vitro or in vivo as appropriate. Thus, the DNA polymer may be prepared by the enzymatic ligation of appropriate DNA fragments, by conventional methods such as those described by D. M. Roberts et al in Biochemistry 1985, 24, 5090-5098.

15   The DNA fragments may be obtained by digestion of DNA containing the required sequences of nucleotides with appropriate restriction enzymes, by chemical synthesis, by enzymatic polymerisation, or by a combination of these methods.

Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50μl or less with 0.1-10μg DNA.

20   Enzymatic polymerisation of DNA may be carried out in vivo, using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50μl or less.

Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer at a temperature of 4°C to ambient, generally in a volume of 50μl or less.

25   The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982),or in other scientific publications, for example M.J. Gait, H.W.D. Matthes M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.
30   S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams et al., Journal of the American Chemical Society,1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes et al., EMBO Journal, 1984, 3, 801. Preferably an automated DNA synthesizer is employed.

35   The DNA polymer is preferably prepared by ligating two or more DNA molecules which together comprise a DNA sequence encoding the hybrid PA.

The DNA molecules may be obtained by the digestion with suitable restriction enzymes of vectors carrying the required coding sequences.

The precise structure of the DNA molecules and the way in which they are obtained depends upon the
40   structure of the desired hybrid PA product. The design of a suitable strategy for the construction of the DNA molecule coding for the hybrid PA is a routine matter for the skilled worker in the art.

In the preferred embodiment, the DNA molecule may conveniently be prepared by ligating a first DNA molecule encoding kringles 1 to 5 of plasminogen with one or more DNA molecules which, together with said first DNA molecule, encode the B-chain of t-PA or u-PA, such that the ligated molecule codes for the
45   amino acid linking sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA.

By way of example and guidance, two strategies for the construction of DNA molecules coding for two specific hybrid plasminogen activators are described below

50

Strategy A.

The first DNA molecule may be obtained by the digestion of a first vector carrying the required part of the plasminogen coding sequence. Conveniently the native plasminogen sequence is modified to introduce
55   a new NarI site 3' to the K_5^P coding region, preferably at nucleotide position 1758, and any NarI site formerly present in the vector is destroyed. The first vector preferably also carries at least part of the t-PA coding sequence including the SstI site at nucleotide position 1417 (Pennica et al., 1983, op. cit.) in the B-chain coding region and the portion of the B-chain coding region 3' thereof. Digestion of the first vector with

7

restriction enzymes NarI and SstI thus provides a DNA molecule coding for plasminog n kringles 1 to 5 and part of the t-PA B-chain, and carrying v ctor functions.

A second DNA molecule may b obtained by the digestion of a second vector carrying the t-PA B-chain coding sequence. Digestion of the second vector with suitable r striction enzyme(s) such as BanII provides a DNA molecule coding for part of the t-PA B-chain. The Ban II sticky end is compatible with the SstI sticky end of the first DNA molecule.

The first and second DNA molecules are linked via an oligonucleotide linker having suitable sticky ends compatible with the NarI and BanII sticky ends of said DNA molecules, and having a structure such that, upon ligation of the first and second DNA molecules with the linker, the resulting molecule codes for an amino acid linking sequence comprising the t-PA cleavage site between residues 275-276 and the cysteine residue 264 of t-PA. Conveniently, the oligonucleotide linker has the following structure:

```
5' CG CCG TCG ACC TGC GGC CTG AGA CAG TAC AGC
3'.   GGC AGC TGG ACG CCG GAC TCT GTC ATG TCG


   CAG CCT CAG TTT CGC ATC AAA GGA GGG CT 3'
   GTC GGA GTC AAA GCG TAG TTT CCT C       5'
```

The ligation of the first and second DNA molecules with the above linker may be carried out sequentially or, more preferably, in a single step. Ligation of the BanII and SstI sticky ends of the molecules reforms the t-PA B-chain coding region. The resulting sequence at the reconstructed NarI site is believed to be as follows:

```
                     NarI
       5' ...GCG GCG CCG TCG ACC TGC GGC CTG... 3'
       3' ...CGC CGC GGC AGC TGG ACG CCG GAC... 5'
           ala ala pro ser*thr cys gly leu
           542 543 544     263 264 265 266
```

Amino acids marked 542-544 are the C-terminal residues of a peptide sequence comprising residues 1 to 544 of plasminogen. The serine residue marked * may be identified with serine 545 of plasminogen or serine 262 of t-PA. Amino acids marked 263-266 are the N-terminal residues of a peptide sequence comprising residues 263-527 of t-PA.

The said first vector carrying the required part of the plasminogen coding sequence, that is, kringles 1 to 5, and with a NarI site introduced at nucleotide position 1758, may conveniently be obtained by the digestion with restriction enzymes HindIII and AhaII and, separately, AhaII and DdeI of a vector carrying the plasminogen gene, in which any existing vector NarI site has been removed by conventional methods. The HindIII site is located in the 5' untranslated region, and the AhaII site at nucleotide position 1572 and the DdeI site at nucleotide position 1748 are in the kringle 5 coding region of the plasminogen gene. The HindIII-AhaII and AhaII-DdeI fragments may be ligated together and to an oligonucleotide linker having a DdeI sticky end, coding for the remainder of the kringle 5 coding region and containing a NarI site. The linker is preferably also provided with a BglII sticky end, such that the resulting DNA molecule, coding for plasminogen kringles 1 to 5, has a HindIII and a BglII sticky end and may be held in a suitable vector such as pTRE12 (EP-A-0 201 153) between the HindIII and BglII sites thereof, creating vector (A).

In order to introduce the part of the t-PA B-chain coding sequence which is 3' to the SstI site into the vector which carries the plasminogen K₁P-K₅P coding region, the BglII fragment encoding the mature t-PA polypeptide obtained from a suitable vector (B) coding therefor, such as pTRE15 (EP-A-0 201 153), is inserted in the BglII site of the vector (A). The unwanted portion of the resulting vector may then be excised by digestion with restriction enzymes NarI and SstI to yield the said first DNA molecule encoding plasminogen kringles 1 to 5, t-PA B-chain 3' to the SstI site and vector functions.

Th said second vector carrying the r quired part of the t-PA coding sequence, that is, th B-chain, may be for example a vector (B) encoding the mature t-PA polypeptide, such as pTRE15 (EP-A-0 201 153).

Strategy B

The first DNA molecule may be obtained by the digestion of a first vector carrying the required part of the plasminogen coding sequence. Conveniently the native plasminogen sequence is modified to introduce a new NarI site 3' to the K_5^P coding region, preferably at nucleotide position 1758, and any NarI site formerly present in the vector is destroyed. The first vector conveniently also has a BglII site positioned such that digestion of the first vector with restriction enzymes NarI and BglII provides a DNA molecule coding for plasminogen kringles 1 to 5 and carrying vector functions.

A second DNA molecule may be obtained by the digestion of a second vector carrying the u-PA B-chain coding sequence. Conveniently the native u-PA sequence is first modified by the introduction of an SstI site 5' to the u-PA B-chain coding region. The second vector preferably also carries at least part of the u-PA A-chain coding sequence 5' to the B-chain coding sequence and conveniently has a BglII site 3' to the B-chain coding sequence. Digestion of the second vector with restriction enzymes BglII and SstI thus provides a DNA molecule coding for u-PA B-chain and part of the A-chain.

The first and second DNA molecules are linked via an oligonucleotide linker having suitable sticky ends, preferably a 5' NarI sticky end and a 3' SstI sticky end, and having a structure such that, upon ligation of the first and second DNA molecules with the linker, the resulting molecule codes for an amino acid linking sequence comprising the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA. Conveniently, the oligonucleotide linker has the following structure:

```
5'  CG CCT TCA TTT CCC TCC TCT CCT CCA GAA GAG CT  3'

3'     GGA AGT AAA GGG AGG AGA GGA GGT CTT C        5'
```

The ligation of the first and second DNA molecules with the above linker may be carried out sequentially or, more preferably, in a single step. The resulting sequence at the reconstructed NarI and SstI sites is believed to be as follows:

```
                NarI
              ┌──⌃──┐
              |  *  |
5' ... TGT GCG G CG CCT TCA TTT CCC TCC TCT CCT CCA GAA
3' ... ACA CGC CGC GGA AGT AAA GGG AGG AGA GGA GGT CTT
       cys ala ala pro ser phe pro ser ser pro pro glu
       541 542 543 544 545 546 137 138 139 140 141 142
```

```
           SstI
         ┌──⌃──┐
         | * * * |
     GAG CT C AAA ... 3'
     C TC GAG TTT ... 5'
     glu leu lys
     143 144 145
```

Amino acids marked 541-546 are the C-terminal residues of a peptide sequence comprising residues 1 to 546 of plasminogen. Amino acids marked 137-145 are the N-terminal residues of a peptide sequence comprising residues 137-411 of u-PA.

The nucleotides marked * have been substituted for the native nucleotides in the course of creating the NarI and SstI restriction sites, without altering the encoded amino acids.

The said first vector carrying the required part of the plasminogen coding sequence, that is, kringles 1 to 5, and with a Narl site introduced at nucleotide position 1758, may conveniently be obtained as described in strategy A above.

The said second vector carrying the required part of the u-PA coding sequence, that is, the B-chain, and with an SstI site 5′ to the B-chain coding region, may conveniently be obtained by conventional mutagenesis techniques to introduce the SstI site into a vector (c) encoding the u-PA polypeptide, conveniently at nucleotide position 563. The u-PA polypeptide is conveniently held between the HindIII and BglII sites of a suitable vector, such as pTRE12 (EP-A-0 201 153).

The expression of the DNA polymer encoding the hybrid PA in a recombinant host cell may be carried out by means of a replicable expression vector capable, in the host cell, of expressing the DNA polymer. The expression vector is novel and also forms part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment, encode the hybrid PA, under ligating conditions.

The ligation of the linear segment and more than one DNA molecule may be carried out simultaneously or sequentially as desired.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic, such as E. Coli, or eukaryotic, such as mouse C127, mouse myeloma, chinese hamster ovary or yeast. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis et al., cited above. Polymerisation and ligation may be performed as described above for the preparation of the DNA polymer. Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50μl or less with 0.1-10μg DNA.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al., cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as E. coli may be treated with a solution of $CaCl_2$ (Cohen et al, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells.

The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis et al and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The hybrid PA expression product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as E. coli it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium.

The DNA polymer may be assembled into vectors designed for isolation of stable transformed mammalian cell lines expressing the hybrid PA; e.g. bovine papillomavirus vectors or amplified vectors in chinese hamster ovary cells (DNA cloning Vol.II D.M. Glover ed. IRL Press 1985; Kaufman, R.J. et al., Molecular and Cellular Biology 5, 1750-1759, 1985; Pavlakis G.N. and Hamer, D.H., Proceedings of the National Academy of Sciences (USA) 80, 397-401, 1983; Goeddel, D.V. et al., European Patent Application No. 0093619, 1983).

It will be appreciated that, depending upon the host cell, the hybrid PA prepared in accordance with the invention may be glycosylated to varying degrees. Furthermore, as observed by Pohl et.al., Biochemistry, 1984, 23, 3701-3707, varying degress of glycosylation may also be found in unmodified, naturally occurring t-PA. Plasminogen also exhibits varying degrees of glycosylation (Hayes M.L, J. Biol. Chem. 254: 8768, 1979). Mutant forms of the hybrid PA are also contemplated in which glycosylation sites are removed by genetic engineering techniques, for example as taught in EP-A-0238304, EP-A-0225286, DE-3537176, WO 87/04722 or EP-0236289. The hybrid PA of the invention is understood to include such glycosylated variations.

It will also be appreciated that, depending upon xpression conditions, the hybrid PA pr par d in accordance with the invention may exist in the single or two chain forms or mixtures th reof. The invention extends to all such forms.

The hybrid PA of the invention comprises the B-chain of native t-PA or u-PA linked to an A-chain comprising the five kringle domains derived from plasminogen and linking sequence of amino acids comprising residues 264 and 275 of t-PA or residues 158 and 148 of u-PA.

This hybrid PA A-chain may be employed as one chain of a fibrinolytically active hybrid protein such as disclosed in EP-0 155 387. The hybrid A-chain, DNA encoding the hybrid A-chain and a hybrid protein comprising the hybrid A-chain linked to the B-chain of a fibrinolytically active protease, the catalytic site of which is optionally blocked by a removable blocking group, all form part of the invention.

The hybrid A-chain may be prepared by separation from the B-chain thereof by mild reduction. Alternatively the hybrid A-chain may be prepared by expressing DNA coding therefor in a recombinant host cell and recovering the hybrid A-chain product. The hybrid protein comprising the hybrid A-chain linked to the B-chain of a fibrinolytically active protease may be prepared by (a) mixing said A- and B-chains under oxidative conditions; or (b) ligating DNA encoding said A-chain to DNA encoding said B-chain and expressing the ligated DNA in a prokaryote or eukaryote host; and thereafter optionally blocking the catalytic site of the hybrid protein with a removable blocking group. The oxidation and reduction conditions are as generally described in EP-A-0 155 387.

The resulting hybrid protein may be used in any of the methods and compositions described hereinafter for the hybrid PA itself.

The hybrid PA of the invention or conjugate thereof can be further derivatised such that any catalytic site essential for fibrinolytic activity is optionally blocked by a removable blocking group.

As used herein the expression 'removable blocking group' includes groups which are removable by hydrolysis at a rate such that the pseudo-first order rate constant for hydrolysis is in the range of 10^{-6} sec^{-1} to 10^{-2} sec^{-1}, more preferably 10^{-6} sec^{-1} to 10^{-3} sec^{-1}, in isotonic aqueous media at pH 7.4 at 37°C.

Such blocking groups are described in European Patent No.0009879 and include acyl groups such as optionally substituted benzoyl or optionally substituted acryloyl.

Suitable optional substituents for benzoyl blocking groups include halogen, C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkanoyloxy, C_{1-6} alkanoylamino, amino or p-guanidino.

Suitable optional substituents for acryloyl blocking groups include C_{1-6} alkyl, furyl, phenyl or C_{1-6} alkylphenyl.

In a preferred aspect, the removable blocking group is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C.

Preferably the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 2.75×10^{-4} s^{-1}, preferably 6.0×10^{-5} to 2.5×10^{-4} s^{-1}, more preferably 6.0×10^{-5} to 2.0×10^{-4} s^{-1}, still more preferably 6.0×10^{-5} to 1.5×10^{-4} s^{-1} and most preferably 7.0×10^{-5} to 1.5×10^{-4} s^{-1}.

Preferably, the 2-aminobenzoyl group is substituted with a halogen atom in the 4-position.

Preferably, the halogen atom is fluorine, chlorine or bromine.

When the group is further substituted, preferred groups include C_{1-6} alkyl, C_{1-6} alkoxy and C_{1-6} alkenyl substituents in the 3- or 5-positions of the ring.

Preferably, the blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

The abovementioned substituted 2-aminobenzoyl blocking groups may be used to derivatise any plasminogen activator comprising the serine protease domain of t-PA or u-PA. This novel group of blocked enzymes within the broad scope of EP-0009879 but not specifically disclosed therein have been found to undergo regeneration of functional active centres at rates corresponding to pseudo first-order rate constants of 6×10^{-5} to 4×10^{-4} sec^{-1}. The latter range of rate constants is especially appropriate for the provision of fibrinolytic activity during the treatment by thrombolytic agents of acute myocardial infarction.

A suitable detergent for use in the buffer is the product having the tradename Tween 80.

Preferred derivatives of this invention include the following:

4-chloro-2-aminobenzoyl plg 1-544/t-PA 262-527 (two chain) including the glu$_1$ and lys$_{78}$ variants and mixtures thereof, and

4-chloro-2-aminobenzoyl plg 1-541/t-PA 262-527 (two chain) including the glu$_1$ and lys$_{78}$ variants and

mixtur s thereof.

The pr sent invention also provides a process for preparing a derivative of th invention, which process comprises reacting the plasminogen activator with a blocking agent JK in which J is a locating group which mediates binding of the agent to the catalytic site of the enzyme and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups.

Examples of group J include 4-amidinophenyl and 2-chloro-4-amidinophenyl.

Preferred agents JK are 4-amidinophenyl 2'-amino-4'-fluorobenzoate; 4-amidinophenyl 2'-amino-4'-chlorobenzoate and 4-amidinophenyl 2'-amino-4'-bromobenzoate.

The blocking reactions are preferably carried out in aqueous media, at a pH in the range 7.0 to 8.5 and at temperatures in the range 0°C to 30°C.

The preferred concentration range for the blocking agent is 0.01 to 2.0 millimolar, and the preferred enzyme concentration range is 1 to 500 micromolar.

In certain cases, particularly with plasminogen activators such as t-PA and those structurally related to or derived from plasminogen, it is desirable to carry out the blocking reaction in the presence of enzyme solubilising or stabilising additives such as 1-arginine, 1-lysine or 6-aminohexanoic acid. The materials have been found to be compatible with the blocking agents JK.

The blocking agents JK may be prepared by the reaction of the appropriate substituted benzoic acid with the appropriate alcohol in an organic base such as pyridine and in the presence of a condensing agent such as dicyclohexylcarbodiimide. The condensation reaction may be performed without prior protection of the 2-amino substituent in the acid component.

The precursor substituted anthranilic acids may be prepared by a variety of methods known in the art. In general, the relative positions of the desired substitutents will determine the chosen starting material. Where an optional substituent such as methyl or methoxy is required, this is generally present in the starting material. The halo, amino and carboxylic acid groups are successively introduced, as necessary, in any appropriate order, by conventional aromatic substitution. Amino and carboxylic acid groups may be provided in precursor form. Amino groups may be obtained from nitro groups by reduction. Carboxylic acid groups may be obtained from methyl groups by oxidation or from ester or thioester groups by hydrolysis. During successive introduction of substituents, it may be necessary to protect amino and/or carboxylic acid groups.

One preferred route comprises the preparation of an appropriate aminotoluene derivative by reduction of the corresponding nitrotoluene, protection of the amino group (for example by acetylation), oxidation of the methyl function to a carboxylic acid group (for example with potassium permanganate) and removal of the protecting group by hydrolysis.

4-Halo-5-methyl anthranilic acids may be prepared by firstly protecting the carboxylate in 4-amino-m-toluic acid with thionyl chloride dimethylamine (to give the dimethylamide), then nitrating the ring at the 2-position with excess butyl lithium/acetyl nitrate, diazotising the free amino group and substituting a halogen atom using the Sandmeyer reaction, reducing the nitro group to give the amine in the 2-position and finally hydrolysing the dimethylamide to the free acid. The same compounds (or, for example, the corresponding 5-methoxy derivatives) may also be prepared from 4-nitro toluene or 4-nitro anisole by halogenation at the 2-position with chlorine/aluminium chloride, reduction to the amine which is then protected by acetylation, insertion of a carboxyl precursor by electrophilic substitution with aluminium chloride and chloroformate ester or thioester and finally hydrolysis of both acetyl and ester/thioester groups to liberate the unprotected amine and carboxyl functions.

The hybrid PA and derivatives of the invention are suitably administered in the form of a pharmaceutical composition.

Accordingly the present invention also provides a pharmaceutical composition comprising a hybrid PA or derivative of the invention in combination with a pharmaceutically acceptable carrier.

The compositions according to the invention may be formulated in accordance with routine procedures as pharmaceutical compositions adapted for intravenous administration to human beings.

Typically compositions for intravenous administration are solutions of the sterile enzyme in sterile isotonic aqueous buffer. Where necessary the composition may also include a solubilising agent to keep the hybrid PA or derivative in solution and a local anaesthetic such as lignocaine to ease pain at the site of injection. Generally, the hybrid PA or derivative will be supplied in unit dosage form for example as a dry powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of protein in activity units. Where composition comprises a derivative of the invention or where the hybrid PA includes a removable blocking group, an indication of the time within which the free protein will be liberated may be given. Where the protein is to be administered by infusion, it will be

dispensed with an infusion bottle containing sterile pharmaceutical grade 'Water for Injection' or saline. Where the protein is to be administered by injection, it is dispensed with an ampoule of sterile wat r for injection or saline. The injectable or infusable composition will be made up by mixing the ingredients prior to administration.

5   The quantity of material administered will depend upon the amount of fibrinolysis required and the speed with which it is required, the seriousness of the thromboembolic condition and position and size of the clot. The precise dose to be employed and mode of administration must per force in view of the nature of the complaint be decided according to the circumstances by the physician supervising treatment. However, in general, a patient being treated for a thrombus will generally receive a daily dose of from 0.01

10   to 10 mg/kg of body weight, such as 0.10 to 2.0mg/kg, either by injection in for example up to five doses or by infusion.

Within the above indicated dosage range, no adverse toxicological effects are indicated with the compounds of the invention.

Accordingly, in a further aspect of the invention there is provided a method of treating thrombotic

15   diseases, which comprises administering to the sufferer an effective non-toxic amount of hybrid PA of the invention.

In another aspect the invention provides the use of a hybrid PA of the invention for the manufacture of a medicament for the treatment of thrombotic diseases.

The invention also provides a hybrid PA of the invention for use as an active therapeutic substance and

20   in particular for use in the treatment of thrombotic diseases.

The present invention further provides a method of treatment of thromboembolic diseases, in particular acute myocardial infarction, which method comprises administering to the sufferer an effective, non-toxic amount of a derivative of the invention.

The invention also provides the use of a derivative of the invention for the manufacture of a medicament

25   for the treatment of thromboembolic diseases, in particular acute myocardial infarction.

In a further aspect, the invention provides a derivative of the invention for use as an active therapeutic substance, in particular for the treatment of thromboembolic diseases such as acute myocardial infarction.

The following Methods and Examples illustrate the invention.

30   I. General Methods used in Examples

(i) DNA cleavage

In general the cleavage of about 1μg of plasmid DNA or DNA fragments was effected using about 5 units of a restriction enzyme (or enzymes) in about 20μl of an appropriate buffer solution.

35   (ii) Generation of blunt ends: If blunt ends were required they were produced by treating the DNA preparation with DNA Polymerase I, Klenow fragment as described by Maniatis et al, (Molecular Cloning- A Laboratory Manual, Cold Spring Harbor Laboratory, 1982), or alternatively (where indicated) by digestion using Mung Bean nuclease (Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

40   (iii) Generation of 'Sticky ends' using linkers: Short kinased oligonucleotide linkers encoding single or multiple restriction sites were ligated onto blunt ended fragments, the linker(s) was digested with the appropriate restriction endonuclease producing the required 'sticky end' necessary for further manipulation. (Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, 1982)

(iv) Ligation of DNA Fragments: Ligation reactions were carried out as described in Maniatis et al,

45   (Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

(v) Transformation of plasmid DNA into E.coli HB101 or E.coli DH5α cells used competent HB101 or DH5α cells supplied by Gibco BRL (Paisley, Scotland), according to the manufacturers instructions.

(vi) Plasmid preparation: Large scale preparation of plasmid DNA and plasmid mini-preparations were carried out as described in Maniatis et al, (Molecular Cloning - A Laboratory Manual, Cold Spring Harbor

50   Laboratory, (1982)).

(vii) Isolation of DNA fragments from low-melting-point (LMP) agarose gels: DNA fragments were isolated from LMP agarose gels as described by Maniatis et al, (Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). Alternatively the excised gel band was purified using GENECLEAN ™, (Stratech Scientific, London) used according to the manufacturers instructions.

55   (viii) Oligonucleotides: Oligonucleotides were made on Applied Biosystems 381A DNA Synthesizer according to the manufacturers instructions and were kinased as described in Maniatis et al, op. cit. When used in plasmid construction the oligonucleotides were annealed by heating together at 95°C for 5 minutes and cooling slowly to room temperature. The annealed oligonucleotides were then ready for

ligation. If four oligonucleotides were to be annealed the annealing reaction was followed as d scribed above but was carried out as 2 reactions each containing a pair of complementary oligonucleotides. After cooling the 2 reactions were mixed prior to ligation.

(ix) DNA sequencing

(a) Single-strand method

Nucleotide sequence determination was carried out by the Sanger dideoxy chain termination method as described in Sanger, S., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467 using ^{35}S-labelled dATP.

(b) Double-strand method

Sequencing was carried out using 'Sequenase™ (United States Biochemical Corporation) essentially according to the manufacturers instructions.

(x) Transient expression of plasminogen activators from HeLa cells

(a) Small-scale

Cell preparation: cells were trypsinised and plated out at approx. 2.4 x 10^5 cells per 35mm dish and incubated in 1.5ml growth medium (this is Hepes buffered RPM1 1640 medium (041-2400) containing 10% Serum (021-6010), 1% stock solution of penicillin/streptomycin (043-5070), 2% sodium bicarbonate solution (043-5080), 1% stock Glutamine (043-5030); Gibco, Paisley, Scotland) at 37°C in a humidified incubator in an atmosphere of 5% CO$_2$/95% air. After 72h the cells were refed, and used for transfection 24h later.

Transfection procedure: Cultures were changed to Eagles MEM (041-1095), 10% serum, 1% penicillin/streptomycin, 1% Glutamine and 1% non-essential amino acids (043-1140) 3h before transfection. The transfections used calcium coprecipitation as described in 'DNA Cloning' Ed. D.M. Glover (Chap. 15, C. Gorman). Glycerol shock and 5mM butyrate treatments were used. Plasminogen activator(s) secreted by transfected cells was harvested in 1.0ml RPMI 1640 medium (as above but serum-free, containing 4% soybean peptone (Sigma) for 24h and activity was measured using fibrin plates with reference to normal t-PA (J.H. Robinson, Thromb. Res., 1984; 33, 155).

Alternatively the HeLa cells were plated at 5 x 10^5 per dish, in which case the cells were refed after only 24h and then used as above.

(b) Large-scale

Cell preparation: cells were trypsinised and plated out at a density of approx. 2.5 x 10^6 cells per 175cm^2 flask in 30ml growth medium (above). After 72h an extra 25ml of growth medium was added and the cells were used for DNA transfection 24h later (as above). 25ml of harvest medium were used per flask.

Alternatively the cells were plated at a density of approximately 20 x 10^6 cells per flask and 25ml of growth medium were added after 96h incubation and the cells used as above.

The two seeding rates and feed times used in the small and large-scale protocols were designed to allow convenient timing of experiments. Both sets of protocols allowed efficient expression of activator(s).

(xi) Western blotting/Immunogold silver staining

Following SDS PAGE, proteins were blotted onto nitrocellulose (NC) by electrophoretic transfer overnight at 4°C,40V, 100mA in 25mM Tris/192mM Glycine/20% (v/v) Methanol pH 8.3. Unreacted sites on the NC were blocked with 5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) pH 7.2 for 1h, followed by a 2h incubation with rabbit anti-t-PA B chain IgG (48μg/ml) in 1% BSA/0.5% Tween 80/PBS pH 7.2 (diluent). The NC was washed, 3 x 5 mins, in 0.5% Tween 80/PBS pH 7.2 and then incubated for 1h in biotinylated secondary antibody (Janssen; 2μg/ml in diluent). The NC was washed as above and incubated in Auroprobe™ BL plus streptavidin (Janssen) 1:100 in diluent for 2h. The NC was washed as above, followed by 1x1 min in deionised water, and silver enhanced using IntenSE™ II kit (Janssen) for 15-20 min. All steps were carried out at ambient temperature with gentle shaking.

(xii) Rate constant determinations

The pseudo first order rate constant is determined by hydrolysing the acyl-enzyme under physiological conditions, i.e. in isotonic aqueous media at pH 7.4 and at 37°C. At regular intervals aliquots are withdrawn and incubated with a chromogenic substrate and the rate of conversion of the substrate measured as indicated above.

The hydrolysis is followed until such time as the rate of conversion of substrate reaches a maximum. The rate constant k may then be calculated by plotting:

Log_e (1-A$_t$/A$_{max}$) against t

where A_{max} is the maximum rate at which an aliquot conv rts substrate and A_t is the rate at which an aliquot converts substrate at time t.

Preferably such rate constants ar calculated by computerised non-linear regression analysis, fitting the A_t and time data to the equation:

$$A_t = A_o + (A_{max} - A_o)(1-e^{-kt})$$

where A_o is the activity of the acyl-enzyme preparation before deacylation.

II. Identification of nucleotides, amino acid residues, N-termini, protein domains and chain nature in the examples

(i) Sequences

All t-PA numbering as in Pennica et al (1983) op. cit.; plasminogen amino acid numbering based on Sottrup-Jensen et al (1978) Atlas of Protein Sequence and Structure Vol. 5, Suppl. 3, p91, National Biomedical Research Foundation, Silver Spring, MD., but updated to include the extra amino acid residue indentified by Forsgren, M. et al (1987) FEBS Letters, 213, 254-260. Plasminogen nucleotide sequences as in Forsgren et al. (op. cit.). All urokinase numbering corresponds to Holmes et al., (1985) Bio Technology 3, 923-929.

(ii) N-termini

1. t-PA:

The mature N-terminus is normally believed to be equivalent to ser_1 (Pennica et al., 1983). Other forms also exist e.g. gly_{-3} or val_{+4} (Pohl et al., 1986 FEBS letters, 168 29-32).

2. plasminogen:

glu_1 indicates the protein is believed to comprise the native (nascent) plasminogen N terminus i.e. amino acid residues 1 onwards.

lys_{78} indicates the protein is believed to comprise the processed lys_{78} N terminus. Alternative processed N-termini e.g. met_{69} and val_{79} are known in nature (Miyashita et al., 1988).

(iii) Protein Domains

The protein domains described in the examples have been abbreviated for convenience and are defined as follows:

1. t-PA:

F^t =amino acid residues   6 to 43 inclusive

G^t =   ''   51 to 84   ''

B^t =   ''   276 to 527   ''

2. plasminogen:

K_1P=   ''   84 to 162 inclusive

K_2P=   ''   166 to 243   ''

K_3P=   ''   256 to 333   ''

K_4P=   ''   358 to 435

K_5P=   ''   462 to 541   ''

the 5 plasminogen
kringles K_1-K_5=   ''   84 to 541   ''

(iv) Chain nature

sc, indicates that the protein is in single chain form.
tc, indicates that the protein is in two chain form.

(v) Vectors

pTRE12 -(EP-0201153)-basic expression vector
pTRE15 -(EP-0201153)-encodes wild-type t-PA
pTRE24 -(EP-0207589)-encodes des(cys$_{51}$-asp87)t-PA
pTRE6F-(EP-0241208)-encodes t-PA finger domain

III. Construction and expression of hybrid proteins

The expression of the proteins of Examples 1 to 7 is carried out in Hela cells.

Example 1

Plasminogen 1-544/t-PA 262-527 (H204)

1.1 Construction of a plasmid carrying plasminogen cDNA

A plasminogen cDNA clone was obtained from a human liver cDNA library after screening with an oligonucleotide probe of sequence (A):
5' CC CCT CAC ACA CAT AAC AGG 3'   (A)
corresponding to nucleotides 49 to 68 of the sequence described in Malinowski et al Biochemistry 23, p4243 (1984).
The DNA sequence of the portion of the plasminogen cDNA clone coding for the A chain of the protein was determined and two differences to the plasminogen cDNA sequence of Forsgren et al (1987) were noted.
The nucleotide at position 846 is T, the nucleotide at position 948 is G.
The 5' end of the clone was shown to correspond to nucleotide 27 of the sequence of Forsgren et al - (1987).

Plasminogen cDNA was subcloned into the vector pUC8 (Vieira and Messing, 1982, Gene 19, 259) between the HincII and EcoRI sites to create plasmid pTRH6 Fig. 1a.

1.2 Modification of pTRE12

The single NarI site formerly in plasmid pTRE12 (EP-A-0 201 153) was destroyed by restriction cutting, blunt ending and religation forming pTRE12D Nar. Removal of the NarI site aided further manipulation.

1.3 Construction of a hybrid plasminogen: t-PA cDNA molecule (plasmid pTRH204)

(All plasminogen nucleotide numbering corresponds to Forsgren et al., 1987, op.cit.)

Three fragments were prepared by restriction digestion and agarose gel electrophoresis. These fragments are as follows:-

I: approximately 1.6kb: from HindIII plus AhaII digest (HindIII [5′ to the plasminogen coding sequence] to AhaII(1572)) of pTRH6.

II: approximately 0.18 kb: from an AhaII plus DdeI digest (AhaII (1572) to DdeI (1748)) of pTRH6.

III: approximately 5.3kb: largest fragment from a HindIII plus BglII digest, of pTRE12D Nar. This fragment carries pTRE12D Nar vector functions.

These fragments (I, II, III) were ligated with a kinased oligonucleotide linker (linker IV) comprising two oligonucleotides of sequence:-
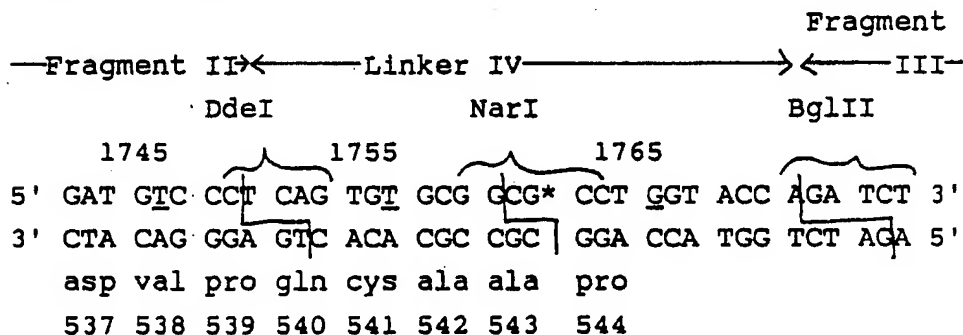
5′ TCAGTGTGCGGCGCCTGGTACCA 3′   (B)

5′ GATCTGGTACCAGGCGCCGCACAC 3′   (C)

After transformation into E.coli HB101 the plasmid pTRH9 (Fig. 2) was isolated.

The DNA in plasmid pTRH9 encodes most of the plasminogen-A chain, up to and including proline 544 i.e. kringles 1 to 5; the DNA encoding the plasminogen B-chain and the remainder of the A-chain were absent. Use of linker IV in construction of pTRH9 introduces a unique NarI site, as may be seen from Table 1 (which shows the junction of fragments II and III and linker IV).

TABLE 1

Junction of fragments II and III and linker IV in plasmid pTRH9.

```
                                                         Fragment
   ─Fragment II><──────Linker IV────────────> <─────III─
        · DdeI                  NarI               BglII
     1745         1755              1765
   5′ GAT GTC CCT CAG TGT GCG GCG* CCT GGT ACC AGA TCT 3′
   3′ CTA CAG GGA GTC ACA CGC CGC | GGA CCA TGG TCT AGA 5′
      asp val pro gln cys ala ala  pro
      537 538 539 540 541 542 543  544
```

The plasminogen amino-acid sequence is shown

*This residue is C in plasminogen cDNA
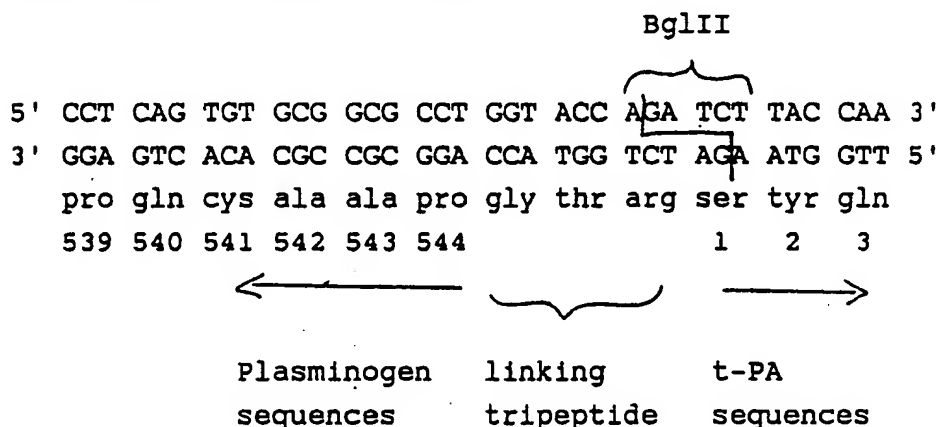
Nucleotide numbering is included (above the sequence)

Plasmid pTRH9 was restricted with BglII and ligated with the 2kb BglII fragment encoding the mature t-PA polypeptide isolated from PTRE15 (EP-A-0 201 153).

An E.coli HB101 clone carrying the t-PA insert in the same orientation (5′->3′) as the plasminogen 'A' chain DNA was isolated; this plasmid was called pTRH20 (see Fig. 3).

Plasmid pTRH20 encodes a molecule comprising the five kringles of plasminog n (residues 1 to 544) linked to the N-terminus of mature t-PA via the tripeptide gly-thr-arg. The junction of the plasminogen A-chain DNA and t-PA A-chain DNA of plasmid pTRH20 is shown in Table 2.

TABLE 2

Junction of the plasminogen A-chain DNA and t-PA A chain DNA in plasmid pTRH20.

```
                                      BglII
                                    ⌒‿‿‿⌒
 5' CCT CAG TGT GCG GCG CCT GGT ACC AGA TCT TAC CAA 3'
 3' GGA GTC ACA CGC CGC GGA CCA TGG TCT AGA ATG GTT 5'
    pro gln cys ala ala pro gly thr arg ser tyr gln
    539 540 541 542 543 544             1   2   3

         ⟵―――――――――――――    ‿‿‿‿‿    ―――――⟶

    Plasminogen       linking      t-PA
    sequences         tripeptide   sequences
```

The DNA encoding the t-PA A-chain and part of the B-chain was removed from pTRH20 by restriction with NarI and SstI (located at t-PA nucleotide 1417 as described by Pennica et al 1983). The large SstI - NarI fragment (7.9kb) was isolated (fragment V).

An approximately 0.4kb BanII fragment (fragment VI) was isolated from pTRE15. The two relevant BanII sites are located at nucletotides 1024 and 1417 in the t-PA cDNA sequence described by Pennica et al - (1983). BanII has a degenerate recognition site i.e.:

5' .... G Pu GC Py C.....3'
3' .... C Py CG Pu G.....5'

Note that the BanII site at 1417 is identical to the SstI site at 1417 and also that the sticky end created is compatible with an SstI sticky end. The 0.4kb BanII fragment encodes most of t-PA B-chain.

Two oligonucleotides (D) and (E):

    5'   CGCCGTCGACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATC

    AAAGGAGGGCT   3'   (D)

    5'   CTCCTTTGATGCGAAACTGAGGCTGGCTGTACTGTCTCAGGCCGCAGGTC

    GACGG   3'   (E)

were synthesized, kinased and combined to form linker VII.

Linker VII has a 5' NarI sticky end, compatible with the NarI sticky end of fragment V and a 3' BanII sticky end compatible with the BanII (nucleotide 1024) sticky end of fragment VI.
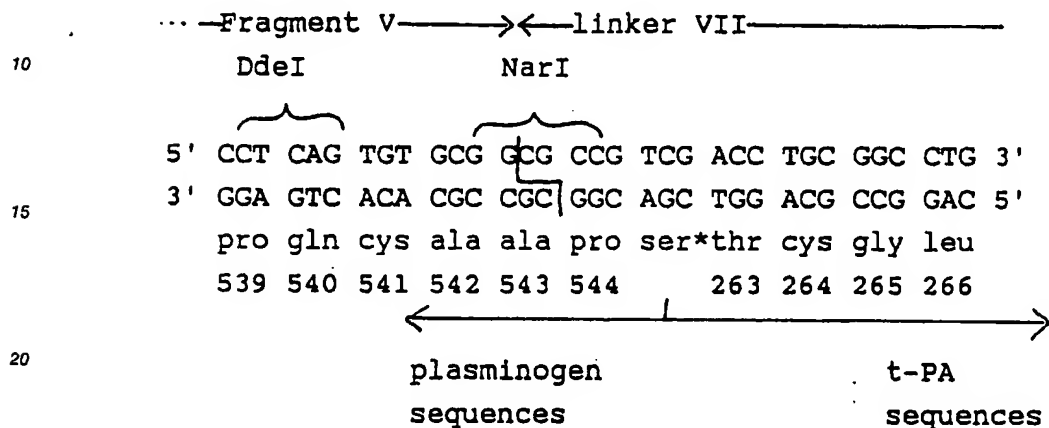
Fragments V and VI together with linker VII were ligated and transformed into E.coli HB101. A plasmid (pTRH204) with the structure depicted in Fig. 4 was isolated.

The junction of fragment V and linker VII is shown in Table 3.

Joining of fragments V and VI via the BanII (1417) and SstI sticky ends completed the reconstruction of the t-PA B-chain coding region.

TABLE 3

Junction of fragment V and linker VII in plasmid pTRH204

```
··· —Fragment V————>←—linker VII————————————
        DdeI              NarI

        ⌒—⌒              ⌒——⌒
5' CCT CAG TGT GCG GCG CCG TCG ACC TGC GGC CTG 3'
3' GGA GTC ACA CGC CGC GGC AGC TGG ACG CCG GAC 5'
   pro gln cys ala ala pro ser*thr cys gly leu
   539 540 541 542 543 544     263 264 265 266
            ←————————————|————————————————>

            plasminogen          t-PA
            sequences            sequences
```

The plasminogen and t-PA amino-acid sequences at the junction are shown (plasminogen numbering 539-544 and t-PA numbering 263-266). The serine residue denoted * may be considered to be equivalent to plasminogen serine 545 or t-PA serine 262.

Plasmid pTRH204 was used to transfect human HeLa cells (as described in Methods) and produced a novel plasminogen activator (H204) believed to have the structure plasminogen 1-544/t-PA 262-527.

1.4 Alternative procedure for plasmid pTRH9

The plasminogen cDNA clone obtained from a human liver cDNA library after screening with oligonucleotide probe (A), is subcloned into the vector pTRE12 (EP-A-0 201 153) between the HindIII and BglII sites to create plasmid pTRH3 Fig. 1(b). The single NarI site formerly in plasmid pTRE12 is destroyed by restriction cutting, blunt ending and religation. Removal of the NarI site aids further manipulation.

Three fragments of pTRH3 are prepared by restriction digestion and agarose gel electrophoresis. These fragments are as follows:-

I: approximately 1.6kb: from a HindIII plus AhaII digest (HindIII [5' to the plasminogen coding sequence] to AhaII[(1572)]).

II: approximately 0.18kb: from an AhaII plus DdeI digest (AhaII [(1572)] to DdeI [(1748)]).

III: approximately 5.3kb: largest fragment from a HindIII plus BglII digest, carries the vector functions of pTRH3.

These fragments (I, II, III) are ligated with the kinased oligonucleotide linker IV described above.

Example 2

Plasminogen 1-544/t-PA 262-527(arg_{275}->gln)(H205)

Construction of plasmid pTRH205

A hybrid plasminogen activator molecule similar to that produced in Example 1 but differing only in the t-PA amino acid residue 275, which is glutamine in place of arginine, also forms part of this invention. The DNA coding for this plasminogen activator was prepared generally as described in Example 1, differing only in the nucleotide sequence of the linker in which the triplet coding for arg-275, CGC, in linker VII was replaced by CAG coding for gln-275. The resulting plasmid pTRH205 was used to transfect human HeLa cells (as described in Methods) and produced a novel plasminogen activator(H205) believed to have the structure plasminogen 1-544/t-PA 262-527 (arg_{275}->gln).

Example 3

Plasminogen 1-541/t-PA 262-527(H37)

5 Construction of plasmid pTRH37

Plasmid pTRH37 was constructed by ligating 2 fragments from pTRH204 (Example 1) together with fragment VI from pTRE15 and an oligonucleotide linker (linker XI):

Fragment IX : 7.2kb fragment of pTRH204 from the SstI site in the t-PA B chain coding region
10 (nucleotide 1417 in the t-PA sequence) to the BstXI site in the plasminogen A chain coding region (nucleotide 1209 in the plasminogen sequence) (Fragment IX thus contains all the vector sequences).

Fragment X : 484 bp fragment of pTRH204 from the BstXI site (1209) to the AvaII site in the plasminogen A chain coding region at nucleotide (1693).

15 Fragment VI : (SstI-BanII) has been described previously (Example 1).

Linker XI was formed by annealing 4 oligonucleotides (H,I,J and K) of sequences:

5' GTCCCTGGTG CTACACGACA AATCCGCGGA AACTTTACGA CTACTGTGAT GTCCCTCAGT GTTCGACCTG CGGCC 3'   (H)

5' TGAGACAGTA CAGCCAGCCT CAGTTTCGCA TCAAAGGAGG GCT 3'   (I)

20 5' CTCCTTTGAT GCGAAACTGA GGCTGGCTGT ACTGTCTCAG GCCGCAGGTC GAACACTGAGGG 3'   (J)

5' ACATCACAGT AGTCGTAAAG TTTCCGCGGA TTTGTCGTGT AGCACCAGG 3'   (K)

A plasmid (pTRH37) was isolated which has the structure shown in Fig 5. The plasmid, when introduced into human HeLa cells, directed the expression of a novel plasminogen activator (H37) which has the same
25 amino-acid sequence as protein H204 (Example 1) except for a deletion of the amino-acid residues alanine (542), alanine (543) and proline (544) immediately C-terminal to the Kringle 5 domain. The hybrid protein structure between Kringle 5 and the protease cleavage site is shown below using single letter notation:

30
$$541^*\qquad\qquad 276$$
$$\underline{C}STCGLRQYSQPQF\underline{R}\underline{I}$$

$$\diagup\qquad\qquad \textbf{t-PA B chain}$$

35
Residue 541 is the last cysteine in Kringle 5. The serine marked with an asterisk can be identified with $serine_{545}$ of plasminogen or $serine_{262}$ of t-PA.

Example 4

40
Plasminogen 78-544/t-PA 262-527(HOO)

4.1 Construction of 'lys$_{78}$-cassette' plasmid

45 The lys$_{78}$-cassette plasmid is a form of the plasmid pTRE12 containing a piece of synthetic DNA that can be used to convert a plasminogen clone from the glu$_1$ form (glu$_1$ is the normal secreted N terminus) to the lys$_{78}$ form (lys$_{78}$ is then the N terminal amino acid).

A pair of kinased oligonucleotide linkers (R + S and T + V) comprising four oligonucleotides:

5' AGCTTCCAGT CCCAAAATGG AACATAAGGA AGTGGTTCTT CTACTTCTTT TATTTCTGAA ATCGG-
50 GACAA GGAAAAGTGT ATCTCT 3'   (R)

5' ACACTTTTCC TTGTCCCGAT TTCAGAAATA AAAGAAGTAG AAGAACCACT TCCTTATGTT CCATTTTGGG ACTGGA 3'   (S)

5' C AGAGTGCAAG ACTGGGAATG GAAAGAACTA CAGAGGTACC ATGTCCAAAA CAAAAAATGG CATCACCTGT CAA 3'   (T)

55 5' GATCTTGACA GGTGATGCCA TTTTTTGTTT TGGACATGGT ACCTCTGTAG TTCTTTCCAT TCCCAGTCTT GCACTCTGAG AGAT 3'   (V)

were prepared and ligated with the large HindIII-BglII fragment from pTRE12.

The resulting plasmid contain d a region form d by the oligonucl otides comprising nucl otid s 65-132 inclusive, joined to nucleotides 364-450 inclusive of native plasminogen. These nucleotides encode the signal sequenc of 19 amino-acids as described by Forsgren et al, (1987) and amino acids 78-106 inclusive of the plasminogen A chain. A unique KpnI site was introduced (Table 4). The structure of the oligonucleotide insert was verified by DNA sequencing.

Table 4

Structure of the lys$_{78}$-cassette insert

```
                       met glu his lys glu val val leu leu
AGCTTCCAGTCCCAAA ATG GAA CAT AAG GAA GTG GTT CCT CTA
HindAGGTCAGGGTTT TAC CTT GTA TTC CTT CAC CAA GAA GAT
III

leu leu leu phe leu lys ser gly gln gly*lys val tyr
CTT CTT TTA TTT CTG AAA TCA GGT CAA GGA AAA GTG TAT
GAA GAA AAT AAA GAC TTT AGT CCA GTT CCT TTT CAC ATA


leu ser glu cys+lys thr gly asn gly lys asn tyr arg
CTC TCA GAG TGC AAG ACT GGG AAT GGA AAG AAC TAC AGA
GAG AGT CTC ACG TTC TGA CCC TTA CCT TTC TTG ATG TCT


gly thr met ser lys thr lys asn gly ile thr cys gln
GGT ACC ATG TCC AAA ACA AAA AAT GGC ATC ACC TGT CAA|BglII
CCA TGG TAC AGG TTT TGT TTT TTA CCG TAG TGG ACA GTT CTAG|
KpnI                                          SfaNI
```

The HindIII and BglII sticky ends were used to clone into the HindIII-BglII sites in pTRE12. SfaNI cuts at a site remote to its recognition sequence. gly* indicates the last residue of the signal sequence; cys+ is the first residue of the K$_1$P domain.

4.2 Construction of plasmid pTRH34 (Plasminogen 1-561/t-PA 276-527)

Plasmid pTRH34 was constructed essentially as described for plasmid pTRH204 (Example 1) by ligation of fragments V and VI together with a linker, but in place of linker VII, linker VIII was used. Linker VIII was formed by annealing 2 oligonucleotides (F and G):
5' CGCCTTCATT TGATTGTGGG AAGCCTCAAG TCGAGCCGAA GAAATGTCCC GGGAGGATCA AAG-GAGGGCT 3'   (F)
5' CTCCTTTGAT CCTCCCGGGA CATTTCTTCG GCTCGACTTG AGGCTTCCCA CAATCAAATG AAGG 3'   (G)
A plasmid pTRH34, with the structure shown in Fig. 6 was isolated. Plasmid pTRH34 when used to transfect human HeLa cells directs the synthesis of a novel plasminogen activator comprising the entire plasminogen A chain (glu$_1$-arg$_{561}$) linked directly to the complete t-PA B chain (ile$_{276}$-pro$_{527}$).

4.3 Construction of plasmid pTRH00

Plasmid pTRHOO is a form of th plasmid pTRH204 (Example 1) in which th nucleotide sequence coding for amino acids glu_1-lys_{77} of th plasminogen protein has been deleted.

5   The following DNA fragments were·prepared by restriction endonuclease digestion and agarose gel electrophoresis:-

Fragment I :- approximately 160bp; from a HindIII plus SfaNI digest of the lys_{78}-cassette plasmid of Example 4.1 [i.e. from the HindIII site at the 5' end of the oligonucleotide linker sequence to the internal SfaNI site - see Table 4] and carries a nucleotide sequence coding for the signal sequence of 19 amino-

10   acids as described by Forsgren et al (1987) joined to amino acids 78-104 inclusive (the SfaNI overhang extends to amino-acids 105 and 106) of the plasminogen A chain.

Fragment II :- approximately 772bp; from a SfaNI plus BstXI digest of pTRH34 of Example 4.2 (SfaNI-(437) to BstXI(1209)), this carries most of $K1^P$, all of $K2^P$ and $K3^P$ and the N-terminal section of $K4^P$.

Fragment III:- approximately 7Kb; from a BstXI and HindIII digest of pTRH204 (BstXI cuts at (1209) and

15   HindIII is located 5' to the plasminogen coding sequence). This carries most of $K4^P$, all of $K5^P$, B^t and the vector functions of pTRE12.

These fragments were ligated, after transformation into E.coli HB101 the plasmid pTRH00 was isolated (Fig. 7). pTRH00 was used to express the novel hybrid plasminogen activator HOO in HeLa cells.

20   ## Example 5

t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-pA 262-527 (HO1)

Plasmid pTRH01 was designed to encode a mature protein in which the F^t coding domain from t-PA is

25   joined to the 5' end of the $K1^P$ coding domain in plasmid pTRH00 (Example 4); the gross domain structure of the encoded native protein can be abbreviated as below:

$$F^tK_1{}^PK_2{}^PK_3{}^PK_4{}^PK_5{}^PB^t$$

30   ### Construction of plasmid pTRH01

Two DNA fragments were prepared by restriction endonuclease digestion and agarose gel electrophoresis. These fragments were as follows:-

Fragment I :- approximately 360bp: from a HindIII plus BamHI digest of plasmid pTR6F (disclosed in EP-

35   0241208). The insert in pTR6F carries the 5' untranslated region and the signal/pro regions as well as the F^t domain.

Fragment II :- approximately 8kb: from a HindIII plus KpnI digest of plasmid pTRH00 (Example 4); this carries almost all of $K1^P$, all of $K2^P$, $K3^P$, $K4^P$,$K5^P$, B^t and the vector functions of pTRE12.

These fragments were ligated with a kinased oligonucleotide linker comprising two oligonucleotides (W

40   and X) of sequence:-

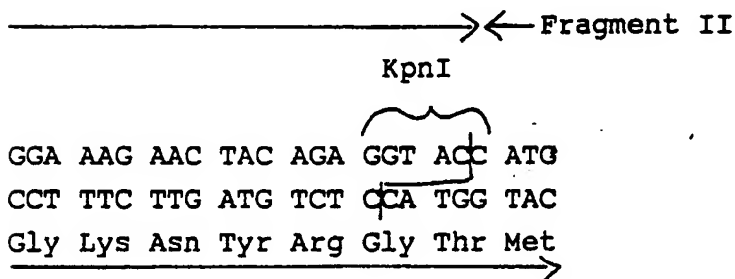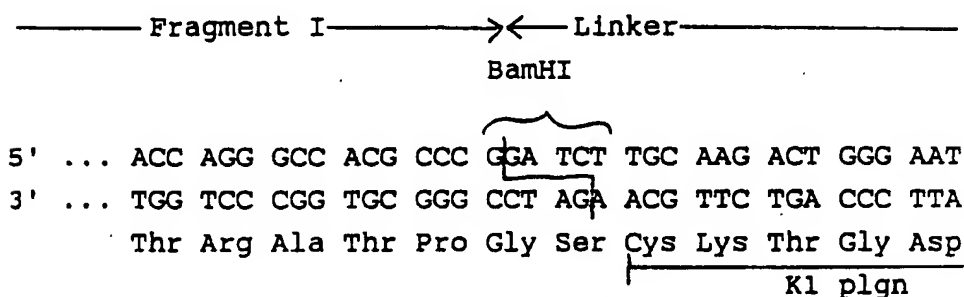5' GATCTTGCAAGACTGGGAATGGAAAGAACTACAGAGGTAC 3'   (W)

5' CTCTGTAGTTCTTTCCATTCCCAGTCCTGCAA 3'   (X)

After transformation into E.coli the plasmid pTRH01 (Fig. 8) was isolated. DNA sequencing confirmed the structure of the F^t/$K1^P$ join (Table 5).

45   pTRH01 was used to express a novel hybrid plasminogen activator (HO1), believed to have the sequence t-PA 1-50/t-PA88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527, in HeLa cells.

50 .

55

Table 5

Junction of Fragments I and II and linker (W + X) in plasmid pTRH01

```
———————— Fragment I ————————>←——Linker———————————
                                    BamHI
```

```
5' ... ACC AGG GCC ACG CCC GGA TCT TGC AAG ACT GGG AAT
3' ... TGG TCC CGG TGC GGG CCT AGA ACG TTC TGA CCC TTA
        Thr Arg Ala Thr Pro Gly Ser Cys Lys Thr Gly Asp
                                          K1 plgn
```

```
————————————————————————————>←——Fragment II
                    KpnI
```

```
GGA AAG AAC TAC AGA GGT ACC ATG
CCT TTC TTG ATG TCT CCA TGG TAC
Gly Lys Asn Tyr Arg Gly Thr Met
————————————————————————————————>
```

Example 6

t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (HO2)

Plasmid pTRH02 was designed to encode a mature protein in which the F^1 and G^1 coding domains from t-PA are joined to the 5' end of the $K1^P$ coding domain in plasmid pTRH00 (Example 4); the gross domain structure of the encoded native protein can be abbreviated as below:

$F^1G^1K_1{}^PK_2{}^PK_3{}^PK_4{}^PK_5{}^PB^1$

6.1 Preparation of DNA encoding the t-PA finger and growth factor domains (plus the t-PA signal/pro and 5' untranslated regions)

6µg of the plasmid pTRE15 (described in EP-A-O 201 153) was digested with MaeII. The MaeII cut

DNA was flush-ended and kinased BamHI linkers (0.1 A_{260} units; Amersham DC1203) of sequence 5'CCCGGATCCGGG3' were ligated to 5µg of the flush-ended MaeII fragments. After ligation excess linkers were removed and cohesive 'sticky-ends' created by digestion with BamHI. The BamH1 digest was electrophoresed on a 1% low melting point agarose gel. On this gel there was a group of three bands with mobility between that of the 1353 and 872 base pair fragments found in a lambda DNA-HindIII/ X174RF-

HaeIII digest (Drigest TMIII, Pharmacia). The largest of these three bands, approximately 1200bp, was recovered (no special attempt was made to ensure that the other bands of similar mobility did not contaminate this preparation, since undesired molecules were eliminated in the cloning strategy). The 1200bp fragment was digested with HindIII to create the desired approx. 470bp fragment with a HindIII 5'

sticky end and a 3' BamHI sticky end (converted from the MaeII site).

This finger-plus-growth-factor domain encoding DNA was sub-cloned into plasmid pTRE12 (EP-A-0207589) between the HindIII and BamHI sites to create plasmid pTR6FG. PTR6FG also carries the 5' untranslated region and signal/pro regions.

6.2 Construction of pTRH02

Two DNA fragments were prepared by restriction endonuclease digestion and agarose gel electrophoresis. These fragments were as follows:

Fragment I : approximately 470bp: from a HindIII plus BamHI digest of plasmid pTR6FG.

Fragment II : approximately 8kb: from a HindIII plus KpnI digest of plasmid pTRH00 (Example 5), carries almost all of K1^P, all of K2^P, K3^P, K4^P, K5^P, B^t and the vector functions of pTRE12.

These fragments were ligated with a kinased oligonucleotide linker comprising two oligonucleotides (W) and (X) of Example 5.

After transformation into E.coli the plasmid pTRH02 was isolated (Fig. 9).

Plasmid pTRH02 was used to express a novel hybrid plasminogen activator (H02), believed to have the sequence t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527, in HeLa cells.

Example 7

Plasminogen 1-546 /u-PA 137-411(H25)

Construction of plasmid pTRH25

A human urokinase cDNA clone is identified by oligonucleotide probing and is isolated by conventional means (Holmes et al, (1985) Bio Technology 3, 923-929). The cDNA clone is modified by conventional in vitro mutagenesis techniques to incorporate an SstI site at nucleotide 563 and a BglII site in the 3' untranslated region. Amino acids 143 and 144 are then coded by the nucleotide triplets GAG CTC rather than the native GAA TTA (plasmid pTRU2).

To create the hybrid enzyme coding sequence, plasmid pTRH9 (Example 1) is digested with NarI and BglII and the approximately 7kb fragment I (carrying vector functions and most of the plasmin A chain coding region) is isolated. Plasmid pTRU2 is digested with SstI and BglII and the urokinase B-chain coding fragment II isolated. An oligonucleotide linker III composed of two kinased oligonucleotides Y and Z is made.

```
5'  CG CCT TCA TTT CCC TCC TCT CCT CCA GAA GAG CT   3'
                                                    (Y)
3'     GGA AGT AAA GGG AGG AGA GGA GGT CTT C   5'   (Z)
```

Fragments I and II and linker III are ligated and transformed into E. coli HB101 and plasmid pTRH25 isolated (Fig.10). The new plasmid encodes a novel hybrid enzyme. The structure in the region of the join between fragment I, II and linker III is believed to be as shown in Table 6.

Table 6

Nar I

```
        ⌢⋀⌢
5' ... TGT GCG GCG CCT TCA TTT CCC TCC TCT CCT CCA GAA
3' ... ACA CGC CGC GGA AGT AAA GGG AGG AGA GGA GGT CTT
       cys ala ala pro ser phe pro ser ser pro pro glu
       541 542 543 544 545 546 137 138 139 140 141 142
       ⟵─────────────────────┼─────────────────────⟶

       plasminogen sequences     urokinase sequences.
```


Sst I

```
  ⌢⋀⌢
GAG CTC AAA ...
CTC GAG TTT ...
glu leu lys
143 144 145
```


Plasmid pTRH25 is used to transfect human Hela cells and produce a novel plasminogen activator.

Example 8

Expression of plasminogen 1-544/t-PA 262-527 (H204) in Chinese Hamster Ovary cells

The plasmid pSV₂dhfr was obtained from the American Type Culture Collection (ATCC 37146 : Molec. Cell. Biol. 1: 854-864, 1981). The plasmid BPV-MT-Xho was obtained from D. Hamer (National Institutes of Health, Bethesda, Maryland) and contains a version of the mouse metallothionein-I gene (Hamer and Walling (1982) J. Mol. Appl. Genet. 1, 273-288) in which the BglII site just 5' to the translation start point has been converted to an XhoI site by linkering. The t-PA mutein-plasmid pTRE24 has been described previously (EP 0 207 589).

8.1 Construction of an amplifiable expression vector:

a. Construction of pTRH69

The plasmid pTRH69 was constructed from two fragments A and B, isolated from the plasmid PSV₂dhfr and the plasmid BPV-MT-Xhol respectiely.

Fragment A:- The plasmid PSV₂dhfr (Fig.11) was linearised with EcoRI, blunt ended by infilling and linkered with Xhol linkers. The linearised plasmid was then digested with Xhol and BglII releasing fragment A, (a 3.4kb fragment encoding the dihydrofolate reductase cDNA and SV₄₀ early promoter sequences).

Fragment B:- The plasmid BPV MT-Xhol was linearised with SstI, blunt-ended by nuclease digestion and linkered using BglII linkers. The linear plasmid was then digested with HindIII, blunt ended by infilling and linkered with Xhol linkers. The plasmid was then digested with BglII and Xhol releasing fragment B (0.3kb encoding the 3' metallothionein polyadenylation sequences) (Fig. 12).

Fragment A and B were isolated by LMP agarose electrophoresis and ligated together. The ligated DNA was tranformed into E.coli HB101 and the plasmid PTRH69 was obtained (Fig. 13).

b. Construction of pTRH71

The plasmid pTRH69 was lin arised with XhoI and phosphatased. The LMP agarose-purifi d fragment was ligated with a 3.3Kb XhoI fragment derived from the plasmid pTRE24 which encodes a modified t-PA protein. The ligated DNA was transformed into E.coli HB101 and the plasmid pTRH71 was obtained (Fig. 14).

c. Construction of pTRH11

The plasmid pTRH71 was digested with MluI and BamHI; a 4.5Kb fragment (comprising the vector functions) was isolated by LMP agarose gel purification and ligated with a 4.1Kb MluI/BamHI fragment isolated from pTRH204 (Example 1) encoding hybrid protein H204. The ligated DNA was transformed into E.coli HB101 and the plasmid pTRH11 was obtained (Fig. 15).

8.2 Expression of hybrid protein

a. Cell preparation

CHO cells were trypsinised and plated out at 5×10^5 per 60mm dish and left in growth medium (Hams F12 nutrient media (041-1765) with 1% stock glutamine (043-5030), 1% stock penicillin/streptomycin (043-5070) and 10% bovine foetal calf serum (013-6290); Gibco, Paisley, Scotland) at 37°C in a humidified incubator in an atmosphere of 5% CO_2/95% air. After 18hrs the cells were re-fed, after a further 3hrs the cells were used for DNA transfection.

b. Transfection procedure

The transfection procedure, carried out in growth medium used calcium coprecipitation and glycerol shock as described in DNA Cloning Ed. D.M. Glover (Chap. 15, C. Gorman). Following transfection the cells were maintained in growth medium for 46hrs under growth conditions (as above) prior to the selection procedure.

c. Selection and Co-amplification

The selection and co-amplification procedure was carried out essentially as described by R.J. Kaufman. (1985) Molecular and Cellular Biology 5, 1750-1759. 46hrs post transfection the cells were medium changed into selective medium (Eagles MEM (041-1095) with 1% stock sodium pyruvate (043-1360), 1% stock glutamine (043-5030), 1% stock penicillin/streptomycin (043-5070) 0.35% proline (22mg/ml) and 10% dialysed bovine foetal calf serum (063-6300) (Gibco, Paisley, Scotland). The cells were maintained in selective medium for 8-10 days until colonies of dhfr+ cells appeared. When colonies were established the cells were medium changed into selective medium containing methotrexate, (A6770; Sigma, England). After 6 days in selective medium containing methotrexate, the selective medium was modified by replacing the Eagles MEM and sodium pyruvate components with α MEM (041-02561). The methotrexate concentration was initially 0.02μM and was increased stepwise to 0.05μM.

d. Detection of hybrid protein H204

During the amplification procedure aliquots of growth medium from growing cells were assayed for plasminogen activator production by fibrin plate and zymography as described by Dodd et al. (1986) Thrombosis and Haemostasis, 55, 94-96. Zymography revealed a fibrinolytically active protein with apparent M_r approximately 100,000, which is consistent with the expected molecular weight of hybrid protein H204.

IV. Purification and post-synthetic modification of hybrid proteins

Exampl A

5   Purification of plasminogen 1-544/t-PA 262-527 (H204)

200ml conditioned harvest media obtained from HeLa cell cultures transfected with the plasmid pTR H204 (Example 1) was centrifuged at 9000g for 30 min and the supernatant retained. It was applied to a column (i.d., 90 mm; h, 220 mm) of Sephadex G25*equilibrated in PBS (Dulbecco A)/0.01% Tween 80 pH 10   7.4 (PBS/TW). The first approx. 0.35 vt was discarded. The following 500ml was retained and was chromatographed on zinc chelate-Sepharose (Vt = 80ml) and lysine Sepharose (Vt = 20ml) essentially as described previously (Dodd, I. et al (1986a) FEBS Lett 209 13). The major difference was that the purified activator was dissociated from the second column using 0.05M sodium phosphate/0.1m sodium chloride/0.01% Tween 80 pH 7.4 (PST) containing 0.02M ϵ-aminocaproic acid (ϵ-ACA). Active fractions 15   eluted by the PST/ϵ-ACA buffer were identified using the chromogenic substrate H-D-ile-pro-arg-p nitroailide (Dodd, I. et al (1986b) Thromb. Haem. 55 94) and were concentrated by stirred-cell ultrafiltration (YM10, Amicon). The ultrafiltered retentate (2.0ml) contained 14400 IU and 4800 SU (Dodd, I. et al 1986b). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) followed by fibrin zymography (Dodd, I. et al. 1986b) showed that the product contained a major fibrinolytic species with apparent M_r approximately 20   100000, consistent with the isolation of a glu$_1$ form of the hybrid activator.

Example B

Synthesis of lys$_{78}$ tc H204 protein (two chain plasminogen 78-544/t-PA 262-527)

25

A preparation of purified H204 protein from Example A that had been buffer-exchanged into PST/10mg ml^{-1} mannitol/50μm ϵ-ACA (89,000 IU, 1.9 ml) was treated with a 0.01 fold molar excess of plasmin at 25°C for 16h and then stored at -40°C. Subsequent analysis of the product showed that it contained 68,000 IU and 32,000 SU. The activator had an apparent M_r of approximately 90,000 by SDS PAGE followed by fibrin 30   zymography. The lys,tc nature of the product was confirmed by SDS PAGE followed by immuno blotting using a polyclonal antibody against the t-PA B chain (see Methods section).

Example C

35   Preparation of 2-Amino-4-chlorobenzoyl plasminogen 78-544/t-PA 262-527 (two chain) ex.Hela cells

Purified lys$_{78}$-tc H204 protein from Example B (3900 SU) in PST/12.5μM ϵ-ACA/2.5mg ml^{-1} Mannitol (1ml) was treated with a 5mM solution of 4-amidinophenyl-2'-amino-4'-chlorobenzoate hydrochloride (Example 11 hereinafter) in dimethylsulphoxide (2μl, 20eq). After 1h incubation at 25°C an inhibition of 40% 40   was noted. A further aliquot (4μl, 40eq) of acylating agent solution was added and after a further 1h at 25°C 93% inhibition had been obtained. The material was buffer exchanged using a Sephadex G25 (PD 10) column into PST/166μM ϵ-ACA/1.66mg ml^{-1} mannitol (1.35ml) and frozen at -40°C.

The material was deacylated by dilution of stock solution (0.3ml) with PST (0.2ml) to give a final solution of PST/100μM ϵ-ACA/1mg ml^{-1} mannitol. The deacylation rate in the above buffer system at 37°C was 45 1.05×10^{-4} sec^{-1} i.e. a t$_{\frac{1}{2}}$ of 110 min.

Example D

Purification of Plasminogen 1-544/t-PA 262-527 (arg$_{275}$->gln)(H205)

50

515ml conditioned harvest medium obtained from HeLa cultures transfected with pTR H205 (Example 2) was purified essentially as described in Example A. The hybrid activator was eluted from the lysine Sepharose column using PST/20mM ϵ-ACA, was concentrated by ultrafiltration and was then buffer-exchanged into PST/10mg ml^{-1} mannitol/50μm ϵ-ACA (2.0ml). The product contained 18,000 IU and 6600 55   SU. Analysis by SDS PAGE followed by fibrin zymography showed the preparation contained two major fibrinolytic species, at apparent M_r approximately 100,000 and 90,000. These are believed to be the glu$_1$

*Sephadex and Sepharose are Trade Marks

and lys$_{78}$ forms of the hybrid activator.

Example E

5   Purification of plasminogen 1-541/t-PA 262-527 (H37)

510ml clarified, conditioned harvest medium that had been obtained from HeLa cell cultures that had been transfected with the plasmid pTR H37 (Example 3) was purified essentially as described for H204 in Example A. The hybrid activator was dissociated from the lysine Sepharose column using PST/20mM ϵ-
10 ACA, was concentrated by ultrafiltration and was then buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ϵ-ACA (2.0ml). The product contained 36,000 IU and 15,000 SU. Analysis by SDS PAGE followed by fibrin zymography showed the preparation contained two major fibrinolytic species, at apparent M$_r$ of approximately 100,000 and 90,000. These are believed to be the glu$_1$ and lys$_{78}$ forms of the activator. SDS PAGE followed by silver staining of non-reduced activator showed the preparation contained major protein bands
15 at M$_r$ approximately 90,000.

Analysis by SDS PAGE followed by immunoblotting using a polyclonal antibody against the t-PA B chain supported the zymography results indicating the presence of glu$_1$ and lys$_{78}$ forms, and, in addition, showed that the product was all two-chain.

20 ## Example F

Preparation of lys$_{78}$ tc H37 protein (two chain plasminogen 78-541/t-PA 262-527)

Purified H37 protein (0.28ml, 7400 SU/ml) prepared as described in Example E, containing equal
25 amounts of the glu$_1$ and lys$_{78}$ forms, was treated either with or without an approx. 0.03 fold molar excess of plasmin at 37°C for 17h. The amidolytic activity (SU) of both products was the same as that of a control preparation that had been stored at -40°C. Analysis by SDS PAGE followed by fibrin zymography showed that the plasmin-treated H37 protein, and, to a lesser extent, the non-plasmin treated H37 protein, had been converted to the lys$_{78}$ form.

30

Example G

Synthesis of 2-amino-4-chlorobenzoyl-plasminogen 1-541/t-PA 262-527 (two chain) and -plasminogen 78-541/t-PA 262-527 (two chain)

35

Purified H37 protein from Example F (10nmoles) in PST/10mg ml^{-1} mannitol/50μM ϵ-ACA (2.0ml) was treated with 4-amidinophenyl-2'-amino-4'-chlorobenzoate.HCl(2μmoles, 40μl) at 25°C. After 60 min the amidolytic activity of the preparation had decreased to <2% of the original activity. The material was buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ϵ-ACA (3.0ml) and stored at -40°C.
40 An aliquot of the acylated product was later diluted with 5 volumes PST/10mg ml^{-1} mannitol/50μM ϵ-ACA and allowed to deacylate at 37°C.

Example H

45 Purification of plasminogen 78-544/t-PA 262-527 (H00)

500ml conditioned harvest medium obtained from HeLa cell cultures transfected with the plasmid pTR H00 (Example 4) was clarified by centrifugation (9000g/30min) and was purified as described for H204 protein in Example A. The hybrid activator was eluted from the lysine Sepharose column (Vt, 49ml) using
50 PST/20mM ϵ-ACA, was concentrated by ultrafiltration and then buffer-exchanged into PST/10mg ml^{-1} mannitol/50μM ϵ-ACA (2.1ml). The product contained 5,700 IU and 2,700 SU. SDS PAGE followed by fibrin zymography indicated the presence of a major fibrinolytic species with apparent M$_r$ of approximately 90,000. SDS PAGE followed by staining for protein or immunoblotting using polyclonal antibody against the t-PA B-chain revealed the material was in the two-chain form. These results are consistent with the isolation
55 of lys$_{78}$ tc hybrid plasminogen activator.

Example I

Purification of t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (H02)

5   380ml Conditioned harvest medium obtained from HeLa cell cultures transfected with the plasmid pTR H02 (Example 6) was clarified by centrifugation (9000g/30min) and purified essentially as described for H204 protein in Example A. The hybrid activator was dissociated from the lysine Sepharose column (Vt, 55ml) using PST/20mM ϵ-ACA. The eluate was concentrated by ultrafiltration and then buffer-exchanged into PST/10mg ml^{-1} mannitol/50µM ϵ-ACA (2.0ml) to give the product. This contained 42,000 IU and 20,400

10   SU. SDS PAGE followed by fibrin zymography showed a major fibrinolytic species at M_r of approximately 100,000. SDS PAGE followed by protein staining or immunoblotting using a polyclonal antibody against the t-PA B-chain showed that the hybrid enzyme was in the two-chain form.

Example J

15

Synthesis of 2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (two chain)

    H02 protein (1ml, 0.25 nmoles) purified as described in Example I was treated with 4-amidinophenyl-2'-amino-4'-chlorobenzoate.HCl (1.25µl, 25nmoles) at 25°C. After 1h 93% of the amidolytic (S2288) activity of

20   the preparation had been lost. After addition of another 25nmoles acylating agent and a further incubation for 15min the material was buffer-exchanged into PST/10mg ml^{-1} mannitol/50µM ϵ-ACA (2.2ml), aliquoted and stored at -70°C.

    Subsequently, an aliquot of the product was diluted with 1 volume 0.1M Tris/0.15M NaCl/20% (v/v) glycerol/0.01% Tween 80 pH 7.4 and allowed to deacylate at 37°C. The rate of deacylation was determined

25   by assaying the S2288 activity of the solution. The deacylation rate constant of the acylated protein under the described conditions was $3.9 \times 10^{-4} \text{sec}^{-1}$.

Example K

30   Purification of t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (H01)

    2.0ml conditioned harvest medium from HeLa cell cultures transfected with the plasmid pTR H01 (Example 5) was buffer-exchanged into PST/10mg ml^{-1} mannitol/50µM ϵ-ACA (3.0ml) and concentrated by ultrafiltration (Centricon*10, Amicon) to about 0.45ml. The concentrate contained 520 IU/ml; analysis by SDS

35   PAGE followed by fibrin zymography revealed a major fibrinolytic species at apparent M_r approx. 100000.

    The hybrid activator was purified by incubating the 0.45ml solution with 0.2ml lysine Sepharose suspension, removing the supernatant, washing the gel with PST and finally eluting the adsorbed activator with PST/20mM ϵ-ACA, to yield about 0.8 ml solution containing 60 IU/ml partially-purified H01 protein.

40   Example L

Purification of plasminogen 1-544/t-PA 262-527(H204) produced by Chinese Hamster Ovary cells.

    CHO cells transfected with pTRH11 (Example 8.1c.) and selected for survival in methotrexate at 0.05

45   µM (Example 8) were harvested (25ml per 175cm^2 flask) for 24h in αMEM (041-02561) containing 1% penicillin/streptomycin (043-5070), 1% glutamine (043-5030) (Gibco, Paisley, Scotland) and 3mM sodium butyrate (9686730F; BDH Chemical Ltd., Poole, England). 1400ml of harvest medium was collected.

    The harvest medium was clarified by centrifugation (9000g/30 min) and purified on zinc chelate Sepharose (Vt, 230 ml) and lysine sepharose (Vt, 46 ml) using essentially the same method as that

50   described for protein H204 in Example A. The major difference was that the lysine Sepharose column was not eluted with the ϵ-ACA- containing buffer, but with the following, in succession:

      a. 0.02MTris/0.5M NaCl/0.01% Tween 80 pH 7.4

      b. as a. but also containing 0.1M L-arginine

      c. as a. but also containing 0.2M L-arginine

55   d. as a. but also containing 0.3M L-arginine

      e. as a. but also containing 0.4M L-arginine

*Centricon is a Trademark

f. as a. but also containing 0.5M L-arginine

Analysis of the individual fractions that had been collected showed that both endogenous CHO-cell t-PA and protein H204 had adsorbed to th lysine Sepharose column, and both had been dissociated by the arginine - containing buffers. Analysis by SDS PAGE followed by fibrin zymography further showed that the majority
5 of purified protein H204 had clearly been separated from the endogenous CHO cell t-PA, the latter being dissociated by a lower concentration of arginine. The apparent M_r of H204 by SDS PAGE followed by fibrin zymography was approx. 100,000 (with a minor species at approx. 90000), clearly different from the endogenous CHO t-PA, at M_r approx. 65000.

Further analysis of H204 protein by Western blotting using a polyclonal antibody against t-PA B-chain
10 showed that the bulk of the protein was in the two chain form.

Example M

Synthesis of 2-amino-4-chlorobenzoyl plasminogen 1-544/t-PA 262-527 and 2-amino-4-chlorobenzoyl plas-
15 minogen 78-544/t-PA 262-527 ex. CHO cells

10,000 IU purified two-chain H204 protein from CHO cells (Example L) in PST/10mgml^{-1} mannitol/50μM ϵ-ACA (1ml) was treated with 4-amidinophenyl-2'-amino-4'-chlorobenzoate.HCl (20mM, 2.5μl) for 1h at 25°C followed by an additional 2.5μl aliquot for a further 20 min. The acylated H204 was buffer-exchanged into
20 PST/10mgml^{-1} mannitol/50μM ϵ-ACA (1.5ml) using Sephadex G25 and aliquoted. Fibrin plate assay indicated that the acyl product contained 3600 IU/ml. The deacylation rate constant was determined as 1.2x10^{-4} sec^{-1}.

Analysis of the acylated product by SDS PAGE followed by fibrin zymography showed two definite species, with apparant M_r approximately 100000 and 90000. The former is likely to be the glu$_1$ form of
25 acylated H204. The latter is most likely the lys$_{78}$ form.

Example 9

4'-Amidinophenyl 2-amino-4-fluorobenzoate

30

(a) 2-Methyl-5-fluoroacetanilide

5-fluoro-2-methylaniline (6.26g, 50mmol) and triethylamine (7.26g, 10ml, 72mmol) were dissolved in dichloromethane (50ml). This mixture was added dropwise to an ice-cooled solution of acetyl chloride
35 (3.925g, 3.55ml, 50mmol) in dichloromethane (100ml) under an atmosphere of nitrogen. The solution was allowed to warm to room temperature and stirred for 3h. The organic layer was then washed successively with 10% sodium hydrogen carbonate solution (50ml), 2M hydrochloric acid (50ml) and saturated sodium sulphate solution (50ml). The organic layer was dried, filtered and evaporated and the residual solid purified by column chromatography (70g silica in dichloromethane -> 10% ethyl acetate/90% dichloromethane) to
40 leave the title compound (5.41g, 65%).

m.p: 127°C ex ethanol and water
^{1}H nmr (CDCl$_3$) δ: 6.5-7.8 (4H, m, aryl-H + N-H), 2.15 (6H,s,CH$_3$ + COCH$_3$)
IR ν_{max} (Nujol):   3270, 1650, 1600, 1530, 1285, 1250, 860, 800 and 610cm^{-1}

45

| Found: | C 64.40 | H 6.26 | N 8.27 |
| C$_9$H$_{10}$NOF requires: | C 64.66 | H 6.03 | N 8.27%. |

50 (b) 2-(Methylcarbonylamino)-4-fluorobenzoic acid

2-Methyl-5-fluoroacetanilide (Example 9(a)) (2.0g, 10mmol) was dissolved in water (50ml) and potassium permanganate (2.25g, 14mmol) was added. The reaction was stirred and heated to reflux until the purple colour disappeared. Two more aliquots of the oxidant (2 x 1.125g) were added at approximately 2h intervals.
55 The reaction was allowed to cool after 6h and the solution was brought to basic pH. It was then filtered through celite and the aqueous solution was acidified. The aqueous layer was extracted with ethyl acetate (50ml) which was then dried, filtered and evaporated to leave the title compound (0.5g, 22%).

m.p: (sublimes above 140°C)from ethanol and water

¹H nmr (CDCl₃) δ: 11.4 (1H,br s, CO₂H), 8.2 (2H, m, aryl-H), 6.8 (1H, m, aryl-H), 2.15 (3H, s, COCH₃)

IR ν$_{max}$ (Nujol):   3470, 3410, 2500-3400, 1705, 1670 and 1615cm⁻¹

Found:	C 54.49	H 4.34	N 6.38
C₉H₃NFO₃ requires:	C 54.83	H 4.09	N 7.10%

(c) 4-Fluoroanthranilic acid

2-(Methylcarbonylamino)-4-fluorobenzoic acid (Example 9(b)) (0.45g 2.3mmol) was suspended in water (15ml) and 5M sodium hydroxide solution was added (1.8ml, 9mmol).

The solution was heated at reflux for 24h and then allowed to cool. The basic solution was extracted with dichloromethane, which was discarded, and acidified. The acidic solution was extracted with ethyl acetate (2 x 25ml), and this was dried, filtered and evaporated to leave the title compound (0.36g, 100%).

m.p: 193-5°C from ethanol and water

¹H nmr (CDCl₃ /d⁶DMSO) δ: 8.0 (4H, m on br hump, aryl-H + 2 x NH and CO₂H), 6.5 (2H, m, aryl-H)

IR ν$_{max}$ (Nujol):   3500, 3380, 2500-3400, 1665, 1600, 1570, 1490, 1225, 1180, 1145, 980, 890, 835, 760 and 620cm⁻¹

Found:	C 54.19	H 3.94	N 8.83
C₇N₆NO₂F requires:	C 54.20	H 3.90	N 9.03%.

(d) 4'-Amidinophenyl 2-amino-4-fluorobenzoate

2-Amino-4-fluorobenzoic acid (Example 9(c)) (320mg) was dissolved in pyridine (2ml) and 4-amidinophenol (364mg) was added. Dicyclohexylcarbodiimide (425mg) was added and the mixture stirred at room temperature under nitrogen for 18h. A mixture of product and dicyclohexylurea was precipitated. This was triturated with ethanol (10ml) and filtered. The product was precipitated by addition of diethylether to the alcoholic solution. The title compound (133mg, 21%) was isolated as flaky white crystals.

m.p: > 250°C from ethanol and ether

¹H nmr (d⁶DMSO) δ: 9.5 (4H, br s, amidine-NH), 8.0 (3H, m, amidine aryl-H + benzoate aryl-H), 7.5 (2H, d, J = 8Hz, amidine aryl-H), 7.0 (2H, s, aryl-NH₂), 6.6 (1H, m, aryl-H), and 6.2 (1H, m, aryl-H)

IR ν$_{max}$ (Nujol):   3470, 3370, 3330, 2500-3500, 1700, 1680, 1630, 1580, 245, 1210, 1180, 1040, 745, 700cm⁻¹

Found:	C 53.76	H 4.23	N 13.35
C₁₄H₁₃N₃O₂FCl. ¼ H₂0 requires:	C 53.51	H 4.33	N 13.37%.

Example 10

4'-Amidinophenyl 2-amino-4-chlorobenzoate

Recrystallized commercial 2-amino-4-chlorobenzoic acid (345mg) and 4-amidinophenol (345mg) were dissolved in pyridine (5ml). Dicyclohexylcarbodiimide (824mg, 2eq) was added and the mixture stirred at room temperature under an atmosphere of dry nitrogen for 3d. In this time a solid was formed. This was isolated by filtration and was found to be a mixture of the title compound and dicyclohexylurea. The material was triturated with chloroform (6 x 10ml) to remove the urea. This left the title compound (81mg, 12%).

m.p: 230-2°C

¹H nmr (d⁶DMSO) δ: 9.20 (4H,s, aryl-amidine-NH), 7.90 (3H, m, aryl-H), 7.52 (2H, d, J = 9Hz, aryl-H), 6.95 (3H, m, aryl-H + NH₂), 6.65 (1H, m, aryl-H)

IR ν$_{max}$ (Nujol):   3460, 3160, 1710, 1680, 1610, 1220, 1180, 1040 cm⁻¹.

Found:	C 51.79	H 4.19	N 12.57
$C_{14}H_{13}N_3Cl_2O_2$ requires:	C 51.55	H 4.02	N 12.88

Example 11

4'-Amidinophenyl 2-amino-4-bromobenzoate

(a) 2-Amino-4-bromotoluene

4-Bromo-2-nitrotoluene (13.5g, 62.5mmol) and granulated tin (11.25g, 95mmol) were placed in a round bottomed flask to which concentrated hydrochloric acid (25ml) was added in small (ca 5ml) aliquots. A gentle reaction was evident during this addition period. When all the acid had been added the reaction mixture was heated and stirred at about 100°C for 4h. The solution was allowed to cool and a solution of sodium hydroxide (18.75g) in water (32ml) was added. The solution was heated at 100°C for 1h. After cooling, water (200ml) was added and the solution was filtered. The aqueous filtrate was extracted with diethyl ether (200ml). The filtered salts were also extracted with diethyl ether (200ml). The combined organic layers were dried, filtered and evaporated to leave an oil. This was purified by distillation (112°C at 4mm Hg) to leave the title compound (6.76g, 58%).

^1H nmr ($CDCl_3$) δ: 6.8 (3H, m, aryl-H), 3.6 (2H, s, NH_2), and 2.1 (3H,s,CH_3).

(b) 2-Methyl-5-bromoacetanilide

This was synthesized in the same manner as the fluoro-analogue (Example 9(a)) in 96% yield from 2-amino-4-bromotoluene (Example 11(a)). The preparation did not require any column chromatography.

m.p: 108-110°C from dichloromethane and 40-60° petroleum ether,
^1H nmr ($CDCl_3$) δ: 7.8 (2H, m, aryl-H + N-H), 7.1 (2H, m, aryl-H), 2.15 (6H, s, CH_3 and $COCH_3$)
IR ν_{max}(Nujol):   3250, 1670, 1650, 1580, 1530, 1400, 1380, 1290, 1230, 1190, 1130, 860, 800, and 700cm^{-1}

Found:	C 48.00	H 4.43	N 6.16
$C_9H_{10}NOBr$ requires:	C 47.39	H 4.42	N 6.14%.

(c) 2-(Methylcarbonylamino)-4-bromobenzoic acid

This material was produced in 10mmol scale from 2-methyl-5-bromoacetanilide (Example 11(b))in the same way as the fluoro-analogue (Example 9(b)). A yield of 58% was obtained.

m.p: 212-5°C from methanol and water
^1H nmr ($CDCl_3$/d^{6}DMSO) δ: 11.4 (2H,br s, NH and CO_2H), 9.3 (1H, m, aryl-H), 8.2 (1H, m, aryl-H), 7.4 (1H, m, aryl-H), 2.3 (3H, s, aryl-CH_3)
IR ν_{max} (Nujol):   3250, 1670, 1650, 1580, 1520, 1290, 860 and 800cm^{-1}

Found:	C 41.43	H 3.08	N 5.31
$C_9H_8BrNO_3$ requires:	C 41.89	H 3.12	N 5.43%.

(d) 4-Bromoanthranilic acid

The title compound was prepared in 48% yield from 2-(methylcarbonylamino)-4-bromobenzoic acid (Example 11(c)) in the same manner as the fluoro-analogue (Example 9(c)).

^1H nmr ($CDCl_3$/d^{6}DMSO) δ: 8.1 (3H, s, CO_2H + NH_2), 7.6 (1H, d, J = 8Hz, aryl-H), 6.8 (1H, m, aryl-H), 6.6 (1H, m, aryl-H).

(e) 4'-Amidinophenyl 2-amino-4-bromob nzoate

2-Amino-4-bromobenzoic acid (Example 11(d)) (0.90g) and 4-amidinophenol (0.718g) were dissolved in pyridine (10ml). Dicyclohexylcarbodiimide (1.71g, 2eq) was added. The reaction was stirred at room temperature under an atmosphere of dry nitrogen for 2d. It was then filtered and the solid obtained was found to be a mixture of the title compound and dicyclohexylurea. The material was repeatedly triturated with chloroform (6 x 20ml) before being isolated by filtration and evaporation. The pure title compound was obtained (144mg, 9%).

^{1}H nmr (d^{6}DMSO) δ: 9.20 (4H, 6r s, amidine-NH), 7.90 (3H, m, aryl-H), 7.55 (2H, d, J = 6Hz, aryl-H), 7.1 (1H, s, aryl-H), 6.95 (2H, br s, NH$_2$), 6.80 (1H, m, aryl-H)

IR ν_{max} (Nujol):   3450, 3200, 1710, 1680, 1610, 1230, 1180, 1040cm^{-1}.

In the figures:
(N.B. Not to scale)

Fig. 1a Structure of pTRH6

■■■ = pUC8 sequences

――― = plasminogen cDNA sequences

←― = restriction sites

Fig. 1b Structure of pTRH3

■■■■ = pTRE12 sequences

――― = plasminogen cDNA sequences (Hind III at 5' end, BGIII at 3' end).

⇦ = restriction sites used in construction

Fig. 2 Structure of pTRH9

Fragments (I) to (III) and linker (IV) are indicated.

■■■ = pTRE12 D Nar Sequences

――― = insert Sequences

PlgnA = plasminogen A chain coding region

⇦ = restriction sites used in construction

←― = other restriction sites

⊢――| = protein coding regions

Fig. 3 Structure of pTRH20

5   �   = vector sequences

         —————   = insert DNA

         ⟨⊐   = restriction sites used in contruction

         ⟵   = other restriction sites

10   *   = linking amino acid sequence

         ⊢——⊣   = protein coding regions

15   Plgn A   = plasminogen A chain-coding region

         t_A   = t-PA A chain coding region

         t_B   = t-PA B chain coding region

20

Fig. 4 Structure of pTRH204

Fragments (V) (Sstl-Narl), (VI)(BanII-Sstl) and linker (VII)(Narl-BanII) are indicated.

25

         ▰▰▰   = vector sequences

         —————   = insert DNA

30   ⟨⊐   = restriction sites used in construction

         ⟵   = other restriction sites

         *   = serine equivalent to plasminogen serine
                        545/t-PA serine 262 at junction

35   ⊢——⊣   = protein coding regions

         PlgnA   = plasminogen A chain coding region
                        (glu-1 to pro-544)

40   t-PA   = t-PA coding region (thr-263 to pro-527)

45

50

55

34

Fig.5 Structure of pTRH37

Fragments (IX)(SstI-BstXI),(X)(BstXI-AvaII) and linker (XI) (AvaII-BanII) are indicated.

▆▆▆ = vector sequences

⎯⎯ = insert DNA

◁▭ = restriction sites used in construction

⟵ = other restriction sites

⊢⎯⊣ = protein coding regions

Plgn = plasminogen coding sequences (glu$_1$-cys$_{541}$)

t-PA = t-PA coding sequences (thr$_{263}$-pro$_{527}$)

* = serine equivalent to plasminogen serine$_{545}$/ t-PA serine$_{262}$ at junction

Fig. 6 Structure of pTRH34

Fragments (V)(SstI-NarI), (VI)(BanII-SstI) and linker (VIII)(NarI-BanII) are indicated.

▆▆▆ = vector sequences ⎯⎯ = insert DNA

◁▭ = restriction sites used in construction

⟵ = other restriction sites

⊢⎯⊣ = protein coding regions

PlgnA = plasminogen A chain coding region (glu-1 to arg-561)

t-PA B = t-PA B chain coding region (ile-276 to pro-527)

Fig. 7 Construction of pTRH00

Fragment I : is a HindIII/SfaNI fragment from the lys$_{78}$-cassette plasmid.

Fragment II : is a SfaNI/BstXI fragment from pTRH34 containing most of K1^P, all of K2^P and K3^P and the N terminal section of K4^P.

Fragment III: is a BstXI/HindIII fragment from pTRH204 containing most of K4^P, all of K5^P, B^t and the vector sequences from pTRE12.

Fig. 8 Construction of pTRH01

Fragment I : is a HindIII/BamHI fragment from pTR6F encoding the t-PA finger domain and associated N-terminal and C-terminal amino acid sequences i.e. [SYQVI] F^t - [HSVPVKSTRATPGS], plus the 5' untranslated region and signal/pro regions.

Fragment II : is a HindIII/KpnI fragment from pTRH00 containing almost all of K1^P, all of K2^P, K3^P, K4^P, K5^P, B^t and the vector functions of pTRE12.

Linker : as described in Example 5.

Fig. 9 Construction of pTRH02

Fragment II : is a HindIII/BamHI fragment from pTR6FG encoding the t-PA F' and G' domains and associated linking N/C terminal sequences i.e. [SYQVI]F'[HSVPVKS]G'[EIDTRATPGS], plus the 5' untranslated region and signal/pro regions.

Fragment I : is a HindIII/KpnI fragment from pTRH00 containing almost all of K1^P, all of K2^P, K3^P, K4^P, K5^P, B' and the vector functions of pTRE12.

Linker (W + X) is as described in Example 6.

Fig.10 Structure of pTRH25

```
Fragment I   = BglII-NarI fragment from pTRH9.
Fragment II  = SstI - BglII fragment encoding urokinase
                 B chain
         III = oligonucleotides Y + Z
         ▬▬▬ = pTRE12 sequences.
         ──── = insert sequences.
         ◄─── = restriction sites used in construction
Plgn          = plasminogen coding region (glu-1 to
                 phe-546).
u             = urokinase coding region (pro-137 to
                 leu-411).
```

Fig. 11

Plasmid PSV$_2$dhfr

```
      P      =  Simian virus polyadenylation ⎞
                sequence                      ⎟
      I      =  Simian virus40 intron         ⎬  SV40 3'
                sequence                      ⎟
     DH      =  dihydrofolate reductase cDNA  ⎠
   SV40E     =  Simian virus40 early promoter
   EcoRI     =  Restriction enzyme site: EcoRI,
                linkered with XhoI linkers.
   BglII     =  Restriction enzyme site: BglII.
```

Fig. 12 Plasmid BPV-MT-Xhol

BPV = Bovine papillomavirus sequences

MT-I = Mouse metallothionein gene sequences - including 3' polyadenylation sequence (0.3Kb fragment B)

SstI = Restriction enzyme site: SstI, linkeredwith BglII linkers.

HindIII = Restriction enzyme site: HindIII, linkered with Xhol linkers.

Fig. 13 Plasmid pTRH69

MMT 3'   = Mouse metallothionein polyad nylation
             sequences: Fragment B excised from plasmid
             BPV-MT-XhoI.

DH   = dihydrofolate reductase cDNA ⎞ Fragment A
SV$_{40}$E   Simian virus$_{40}$ early promoter ⎛excised from
                                             ⎬plasmid
                                             ⎠PSV$_2$dhfr

XhoI   = Restriction enzyme site XhoI
BglII   = Restriction enzyme site BglII

Fig. 14 Plasmid pTRH71

LTR   = Rous sarcoma virus long terminal ⎞
             repeat   ⎛ 3.3Kb
SV$_{40}$3' = Simian virus$_{40}$intron and   ⎛ fragment from
             polyadenylation sequences   ⎬ pTRE24
MUT   = t-PA Mutein cDNA   ⎠
MMT3'   = Mouse metallothionein polyadenylation
             sequences
DH   = dihydrofolate reductase cDNA
SV$_{40}$E   = Simian virus early promoter

Fig. 15 Plasmid pTRH11

LTR   = Rous sarcoma virus long terminal ⎞
             repeat   ⎛ 4.1Kb
SV$_{40}$3' = Simian virus$_{40}$intron and   ⎛ fragment
             polyadenylation sequences   ⎬ from
H204   = H204 hybrid protein cDNA   ⎠ pTRH204
MMT3'   = Mouse metallothionein polyadenylation
             sequences
DH   = Dihydrofolate reductase cDNA
SV$_{40}$E   = Simian virus$_{40}$ early promoter

Claims
Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. A hybrid plasminogen activator which comprises the five kringle domains of plasminogen linked to the B-chain of t-PA or u-PA via an amino acid sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA, said sequence being selected from:

   1. [AAPSTCGLRQYSQPQFR]
   2. [AAPSTCGLRQYSQPQFQ]
   3. [STCGLRQYSQPQFR],

   or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA, said sequence being:
   [AAPSFPSSPPEELKFQCGQKTLRPRFK] (single letter amino acid notation)

2. A hybrid plasminogen activator according to claim 1 of the formula:

   $(Y-)_m (K^P-Z^t-)_5 B^t$

   where B^t comprises residues 276-527 of t-PA, m is 1, each of the 5 values of K^P represents a kringle domain derived from plasminogen in sequence and Y and each of the 5 values of Z^t independently represents a bond or a linking sequence of amino acids which may be introduced synthetically during the preparation of the hybrid plasminogen activator and/or derived from native plasminogen and/or t-PA sequences, the sequence Z_5^t being the amino acid linking sequence defined in claim 1.

3. A hybrid plasminogen activator according to claim 2 wherein Y represents plasminogen residues 1 to 83 or 78 to 83 respectively.

4. A hybrid plasminogen activator according to claim 2 or 3 wherein Z_1^t, Z_2^t, Z_3^t and Z_4^t represent the native plasminogen inter-domain sequences between plasminogen kringle domains 1 and 2, 2 and 3, 3 and 4 and 4 and 5, respectively.

5. Plasminogen 1-544/t-PA 262-527 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof,
   plasminogen 1-544/t-PA 262-527 (arg_{275} -> gln) including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof,
   t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly_{-3}, ser_1 and val_4 variants, and mixtures thereof,
   t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly_{-3}, ser_1 and val_4 variants, and mixtures thereof or
   plasminogen 1-546/u-PA 137-411 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof.

6. Plasminogen 1-541/t-PA 262-527 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof.

7. A hybrid plasminogen activator according to any preceding claims expressed using chinese hamster ovary cells.

8. A hybrid plasminogen activator according to any preceding claim wherein the catalytic site essential for fibrinolytic activity is blocked by a removable blocking group.

9. A hybrid plasminogen activator according to claim 8 wherein the removable blocking group is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups, wherein the

pseudo first order rat constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v deterg nt comprising polyoxyethylenesorbitan monoleate having a molecular w ight of approximately 1300, at pH 7.4 at 37 ˚ C.

10. A hybrid plasminogen activator according to claim 9 wherein the removable blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

11. 2-Amino-4-chlorobenzoyl plasminogen 1-544/t-PA 262-527 (two chain), including glu$_1$ and lys$_{78}$ variantsand mixtures thereof,
2-amino-4-chlorobenzoyl plasminogen 1-541/t-PA 262-527 (two chain), including glu$_1$ and lys$_{78}$ variants and mixtures thereof or
2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (two chain), including gly$_{-3}$, ser$_1$ and val$_4$ variants and mixtures thereof.

12. A pharmaceutical composition comprising a hybrid plasminogen activator according to any preceding claim in combination with a pharmaceutically acceptable carrier.

13. A hybrid plasminogen activator according to any of claims 1 to 11 for use as an active therapeutic substance.

14. A hybrid plasminogen activator according to any of claims 1 to 11 for use in the treatment of thrombotic diseases.

15. Use of a hybrid plasminogen activator according to any of claims 1 to 11 in the preparation of a medicament for the treatment of thrombotic diseases.

16. A process for preparing a hybrid plasminogen activator according to any of claims 1 to 7 which process comprises expressing DNA encoding said hybrid plasminogen activator in a recombinant host cell and recovering the hybrid plasminogen activator product.

17. A DNA polymer comprising a nucleotide sequence that encodes a hybrid plasminogen activator according to any of claims 1 to 7.

18. A process for preparing a derivative according to claim 8 which process comprises blocking the catalytic site essential for fibrinolytic activity in the hybrid plasminogen activator according to any of claims 1 to 7 with a removable blocking group.

19. A process for preparing a derivative according to any of claims 9 to 11 which process comprises reacting the plasminogen activator with a blocking agent JK in which J is a locating group which mediates binding of the agent to the catalytic site of the enzyme and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups.
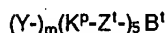
Claims for the following Contracting State : ES

1. A process for preparing a hybrid plasminogen activator which comprises the five kringle domains of plasminogen linked to the B-chain of t-PA or u-PA via an amino acid sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA, said sequence being selected from:

1.	[AAPSTCGLRQYSQPQFR]
2.	[AAPSTCGLRQYSQPQFQ]
3.	[STCGLRQYSQPQFR]

or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA, said

sequence b ing: [AAPSFPSSPPEELKFQCGQKTLRPRFK] (single l tter amino acid notation) and wherein the catalytic site essential for fibrinolytic activity is optionally blocked by a removable blocking group, which process comprises xpressing DNA encoding said hybrid plasminog n activator in a recombinant host cell and recovering the hybrid plasminogen activator product, and thereafter optionally blocking the catalytic site essential for fibrinolytic activity with a removable blocking group.

2. A process according to claim 1 for preparing a hybrid plasminogen activator of the formula:

$$(Y\text{-})_m(K^P\text{-}Z^t\text{-})_5 B^t$$

where B^t comprises residues 276-527 of t-PA, m is 1, each of the 5 values of K^P represents a kringle domain derived from plasminogen in sequence and Y and each of the 5 values of Z^t independently represents a bond or a linking sequence of amino acids which may be introduced synthetically during the preparation of the hybrid plasminogen activator and/or derived from native plasminogen and/or t-PA sequences, the sequence Z_5^t being the amino acid linking sequence defined in claim 1.

3. A process according to claim 2 wherein Y represents plasminogen residues 1 to 83 or 78 to 83 respectively.

4. A process according to claim 2 or 3 wherein Z_1^t, Z_2^t, Z_3^t and Z_4^t represent the native plasminogen inter-domain sequences between plasminogen kringle domains 1 and 2, 2 and 3, 3 and 4 and 4 and 5, respectively.

5. A process according to claim 1 for preparing plasminogen 1-544/t-PA 262-527 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof,
plasminogen 1-544/t-PA 262-527 (arg_{275} -> gln) including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof,
t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly_{-3}, ser_1 and val_4 variants, and mixtures thereof,
t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly_{-3}, ser_1 and val_4 variants, and mixtures thereof or
plasminogen 1-546/u-PA 137-411 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof.

6. A process according to claim 1 for preparing plasminogen 1-541/t-PA 262-527 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof.

7. A process according to any preceding claim wherein the host cell is a chinese hamster ovary cell.

8. A process for preparing a hybrid plasminogen activator according to any preceding claim, wherein the removable blocking group is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C.

9. A process according to claim 8 wherein the removable blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

10. A process according to claim 1 for preparing 2-amino-4-chlorobenzoyl plasminogen 1-544/t-PA 262-527 (two chain), including glu_1 and lys_{78} variants and mixtures thereof,
2-amino-4-chlorobenzoyl plasminogen 1-541/t-PA 262-527 (two chain), including glu_1 and lys_{78} variants and mixtures thereof, or
2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (two chain), including gly_{-3}, ser_1 and val_4 variants and mixtures thereof.

11. A process for preparing a pharmaceutical composition comprising a hybrid plasminogen activator as defined in any preceding claim in combination with a pharmaceutically acceptable carrier which process compris s admixing said activator and said carri r.

12. Use of a hybrid plasminogen activator as defined in any of claims 1 to 10 in the preparation of a medicament for the treatment of thrombotic diseases.

13. A process for preparing a DNA polymer comprising a nucleotide sequence that encodes a hybrid plasminogen activator as defined in any of claims 1 to 6 by the condensation of appropriate mono-, di- or oligomeric units.

14. A process for preparing a derivative of a plasminogen activator as defined in claim 8, which process comprises reacting the plasminogen activator with a blocking agent JK in which J is a locating group which mediates binding of the agent to the catalytic site of the enzyme and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups.

15. A process according to claim 14 wherein the pseudo first order rate constant is in the range 7.0×10^{-5} to 1.5×10^{-4} s^{-1}.

16. A process according to claim 14 or 15 wherein the blocking group is 4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

17. A process according to claim 14 for preparing a derivative as defined in claim 10.

Claims for the following Contracting State : GR

1. A hybrid plasminogen activator which comprises the five kringle domains of plasminogen linked to the B-chain of t-PA or u-PA via an amino acid sequence comprising, respectively, the t-PA cleavage site between residues 275 and 276 and the cysteine residue 264 of t-PA, said sequence being selected from:

    1.   [AAPSTCGLRQYSQPQFR]

    2.   [AAPSTCGLRQYSQPQFQ]

    3.   [STCGLRQYSQPQFR],

or the u-PA cleavage site between residues 158 and 159 and the cysteine residue 148 of u-PA, said sequence being:
[AAPSFPSSPPEELKFQCGQKTLRPRFK] (single letter amino acid notation)

2. A hybrid plasminogen activator according to claim 1 of the formula:

$$(Y\text{-})_m(K^P\text{-}Z^t\text{-})_5 B^t$$

where B^t comprises residues 276-527 of t-PA, m is 1, each of the 5 values of K^P represents a kringle domain derived from plasminogen in sequence and Y and each of the 5 values of Z^t independently represents a bond or a linking sequence of amino acids which may be introduced synthetically during the preparation of the hybrid plasminogen activator and/or derived from native plasminogen and/or t-PA sequences, the sequence Z_5^t being the amino acid linking sequence defined in claim 1.

3. A hybrid plasminogen activator according to claim 2 wherein Y represents plasminogen residues 1 to 83 or 78 to 83 respectively.

4. A hybrid plasminogen activator according to claim 2 or 3 wherein Z_1^t, Z_2^t, Z_3^t and Z_4^t represent the native plasminogen inter-domain sequences between plasminogen kringle domains 1 and 2, 2 and 3, 3 and 4 and 4 and 5, respectively.

5. Plasminogen 1-544/t-PA 262-527 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof,

plasminogen 1-544/t-PA 262-527 (arg_{275} -> gln) including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof,

t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly_{-3}, ser_1 and val_4 variants, and mixtures thereof,

t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 including one and two chain variants, gly_{-3}, ser_1 and val_4 variants, and mixtures thereof or

plasminogen 1-546/u-PA 137-411 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof.

6. Plasminogen 1-541/t-PA 262-527 including one and two chain variants, lys_{78} and glu_1 variants, and mixtures thereof.

7. A hybrid plasminogen activator according to any preceding claims expressed using chinese hamster ovary cells.

8. A hybrid plasminogen activator according to any preceding claim wherein the catalytic site essential for fibrinolytic activity is blocked by a removable blocking group.

9. A hybrid plasminogen activator according to claim 8 wherein the removable blocking group is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups, wherein the pseudo first order rate constant for hydrolysis of the derivative is in the range 6.0×10^{-5} to 4.0×10^{-4} sec^{-1} when measured in a buffer system consisting of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v detergent comprising polyoxyethylenesorbitan monoleate having a molecular weight of approximately 1300, at pH 7.4 at 37°C.

10. A hybrid plasminogen activator according to claim 9 wherein the removable blocking group is4-fluoro-2-aminobenzoyl, 4-chloro-2-aminobenzoyl or 4-bromo-2-aminobenzoyl.

11. 2-Amino-4-chlorobenzoyl plasminogen 1-544/t-PA 262-527 (two chain), including glu_1 and lys_{78} variantsand mixtures thereof,

2-amino-4-chlorobenzoyl plasminogen 1-541/t-PA 262-527 (two chain), including glu_1 and lys_{78} variants and mixtures thereof or

2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogen 84-544/t-PA 262-527 (two chain), including gly_{-3}, ser_1 and val_4 variants and mixtures thereof.

12. Use of a hybrid plasminogen activator according to any of claims 1 to 11 in the preparation of a medicament for the treatment of thrombotic diseases.

13. A process for preparing a hybrid plasminogen activator according to any of claims 1 to 7 which process comprises expressing DNA encoding said hybrid plasminogen activator in a recombinant host cell and recovering the hybrid plasminogen activator product.

14. A DNA polymer comprising a nucleotide sequence that encodes a hybrid plasminogen activator according to any of claims 1 to 7.

15. A process for preparing a derivative according to claim 8 which process comprises blocking the catalytic site essential for fibrinolytic activity in the hybrid plasminogen activator according to any of claims 1 to 7 with a removable blocking group.

16. A process for preparing a derivative according to any of claims 9 to 11 which process comprises reacting the plasminogen activator with a blocking agent JK in which J is a locating group which mediates binding of the agent to the catalytic site of the enzyme and K is a 2-aminobenzoyl group substituted in the 3- or 4-position with a halogen atom and optionally further substituted with one or more weakly electron-withdrawing or electron-donating groups.

Patentansprüche
Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Hybridplasminogenaktivator, der die fünf Kringle-Domänen von Plasminogen umfaßt, die an die B-Kette von t-PA bzw. u-PA über eine Aminosäuresequenz geknüpft sind, die die t-PA-Spaltstelle zwischen den Resten 275 und 276 und den Cysteinrest 264 von t-PA umfaßt, wobei die Sequenz ausgewählt ist aus:

    1.   [AAPSTCGLRQYSQPQFR]

    2.   [AAPSTCGLRQYSQPQFQ]

    3.   [STCGLRQYSQPQFR]

   bzw. die u-PA-Spaltstelle zwischen den Resten 158 und 159 und den Cysteinrest 148 von u-PA umfaßt, wobei die Sequenz ist:
   [AAPSFPSSPPEELKFQCGQKTLRPRFK]
   (Einbuchstabencode für Aminosäuren)

2. Hybridplasminogenaktivator nach Anspruch 1 mit der Formel:

   $(Y-)_m(K^p-Z^t-)_5 B^t$

   wobei B^t die Reste 276-527 von t-PA umfaßt, m 1 ist, die fünf Werte für K^p jeweils eine Kringle-Domäne darstellen und in Folge von Plasminogen abstammen, und Y und jeder der 5 Werte für Z^t unabhängig voneinander eine Bindung oder eine verknüpfende Aminosäuresequenz darstellt, die während der Herstellung des Hybridplasminogenaktivators synthetisch eingeführt werden und/oder von nativem Plasminogen und/oder t-PA-Sequenzen abstammen kann, wobei die Sequenz Z_5^t der in Anspruch 1 definierten verknüpfenden Aminosäuresequenz entspricht.

3. Hybridplasminogenaktivator nach Anspruch 2, wobei Y die Plasminogenreste 1 bis 83 bzw. 78 bis 83 darstellt.

4. Hybridplasminogenaktivator nach Anspruch 2 oder 3, wobei Z_1^t, Z_2^t, Z_3^t und Z_4^t die nativen Plasminogenzwischendomänensequenzen zwischen den Plasminogenkringle-Domänen 1 und 2, 2 und 3, 3 und 4 bzw. 4 und 5 darstellen.

5. Plasminogen 1-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon,
   Plasminogen 1-544/t-PA 262-527 (arg275 → gln), einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon,
   t-PA 1-50/t-PA 88-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon,
   t-PA 1-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon, oder
   Plasminogen 1-546/u-PA 137-411, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon.

6. Plasminogen 1-541/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon.

7. Hybridplasminogenaktivator nach einem der vorstehenden Ansprüche, exprimiert unter Verwendung von Ovarzellen des Chinesischen Hamsters.

8. Hybridplasminogenaktivator nach einem der vorstehenden Ansprüche, wobei der für die fibrinolytische Aktivität essentielle Katalyseort durch eine entfernbare blockierende Gruppe blockiert ist.

9. Hybridplasminogenaktivator nach Anspruch 8, wobei die entfernbare blockierende Gruppe eine 2-Aminobenzoylgruppe ist, die an der 3- oder 4-Position mit einem Halogenatom und außerdem

wahlweise mit einer oder mehreren schwach elektronen ntziehenden oder el ktronenspend nden Gruppe(n) substituiert ist, wob i die Geschwindigkeitskonstante pseudoerster Ordnung der Hydrolyse des Derivats im Bereich von $6,0 \times 10^{-5}$ bis $4,0 \times 10^{-4} \ sec^{-1}$ liegt bei Messung in einem Puffersyst m bestehend aus 0,05 M Natriumphosphat, 0,1 M Natriumchlorid, 0,01 % (Vol./Vol.) Detergens, das Polyoxyethylensorbitanmonooleat mit einem Molekulargewicht von etwa 1300 umfaßt, bei einem pH-Wert von 7,4 bei 37° C.

10. Hybridplasminogenaktivator nach Anspruch 9, wobei die entfernbare blockierende Gruppe 4-Fluor-2-aminobenzoyl, 4-Chlor-2-aminobenzoyl oder 4-Brom-2-aminobenzoyl ist.

11. 2-Amino-4-chlorbenzoylplasminogen 1-544/t-PA 262-527 (Zwei Ketten), einschließlich glu_1- und lys_{78}-Varianten und Gemische davon,
2-Amino-4-chlorbenzoylplasminogen 1-541/t-PA 262-527 (zwei Ketten), einschließlich glu_1- und lys_{78}-Varianten und Gemische davon, oder
2-Amino-4-chlorbenzoyl-t-PA 1-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527 (zwei Ketten), einschließlich gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon.

12. Pharmazeutische Zusammensetzung, die einen Hybridplasminogenaktivator nach einem der vorangegangenen Ansprüche in Verbindung mit einem pharmazeutisch verträglichen Träger umfaßt.

13. Hybridplasminogenaktivator nach einem der Ansprüche 1 bis 11 zur Verwendung als aktive therapeutische Substanz.

14. Hybridplasminogenaktivator nach einem der Ansprüche 1 bis 11 zur Verwendung bei der Behandlung von thrombotischen Krankheiten.

15. Verwendung eines Hybridplasminogenaktivators nach einem der Ansprüche 1 bis 11 zur Herstellung eines Medikaments zur Behandlung von thrombotischen Krankheiten.

16. Verfahren zur Herstellung eines Hybridplasminogenaktivators nach einem der Ansprüche 1 bis 7, wobei das Verfahren die Expression der den Hybridplasminogenaktivator codierenden DNA in einer rekombinanten Wirtszelle und die Gewinnung des Hybridplasminogenaktivatorprodukts umfaßt.

17. DNA-Polymer, das eine Nucleotidsequenz umfaßt, die einen Hybridplasminogenaktivator nach einem der Ansprüche 1 bis 7 codiert.

18. Verfahren zur Herstellung eines Derivats nach Anspruch 8, wobei das Verfahren die Blockierung des für die fibrinolytische Aktivität in dem Hybridplasminogenaktivator nach einem der Ansprüche 1 bis 7 essentiellen Katalyseorts mit einer entfernbaren blockierenden Gruppe umfaßt.

19. Verfahren zur Herstellung eines Derivats nach einem der Ansprüche 9 bis 11, wobei das Verfahren die Umsetzung des Plasminogenaktivators mit einem blockierenden Mittel JK umfaßt, wobei J eine lokalisierende Gruppe darstellt, die die Bindung des Mittels an den Katalyseort des Enzyms vermittelt und K eine an der 3- oder 4-Position mit einem Halogenatom und außerdem wahlweise mit einer oder mehreren schwach elektronenentziehenden oder elektronenspendenden Gruppe(n) substituierte 2-Aminobenzoylgruppe darstellt.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zur Herstellung eines Hybridplasminogenaktivators, der die fünf Kringle-Domänen von Plasminogen umfaßt, die an die B-Kette von t-PA bzw. u-PA über eine Aminosäuresequenz geknüpft sind, die die t-PA-Spaltstelle zwischen den Resten 275 und 276 und den Cysteinrest 264 von t-PA umfaßt, wobei die Sequenz ausgewählt ist aus:

1.   [AAPSTCGLRQYSQPQFR]

2.   [AAPSTCGLRQYSQPQFQ]

3.   [STCGLRQYSQPQFR]

bzw. die u-PA-Spaltstelle zwischen den Resten 158 und 159 und den Cysteinrest 148 von u-PA umfaßt, wobei die Sequenz ist: [AAPSFPSSPPEELKFQCGQKTLRPRFK] (Einbuchstabencode für Aminosäuren) und wobei der für die fibrinolytische Aktivität essentielle Katalyseort wahlweise mit einer entfernbaren blockierenden Gruppe blockiert ist, wobei das Verfahren die Expression der den Hybridplasminogenaktivator codierenden DNA in einer rekombinanten Wirtszelle und die Gewinnung des Hybridplasminogenaktivatorprodukts umfaßt und danach wahlweise die Blockierung des für die fibrinolytische Aktivität essentiellen Katalyseorts mit einer entfernbaren blockierenden Gruppe.

2. Verfahren nach Anspruch 1 zur Herstellung eines Hybridplasminogenaktivators mit der Formel:
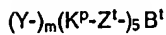
$$(Y\text{-})_m(K^P\text{-}Z^t\text{-})_5 B^t$$

wobei B^t die Reste 276-527 von t-PA umfaßt, m 1 ist, die fünf Werte für K^P jeweils eine Kringle-Domäne darstellen und in Folge von Plasminogen abstammen, und Y und jeder der 5 Werte für Z^t unabhängig voneinander eine Bindung oder eine verknüpfende Aminosäuresequenz darstellt, die während der Herstellung des Hybridplasminogenaktivators synthetisch eingeführt werden und/oder von nativem Plasminogen und/oder t-PA-Sequenzen abstammen kann, wobei die Sequenz Z_5^t der in Anspruch 1 definierten verknüpfenden Aminosäuresequenz entspricht.

3. Verfahren nach Anspruch 2, wobei Y die Plasminogenreste 1 bis 83 bzw. 78 bis 83 darstellt.

4. Verfahren nach Anspruch 2 oder 3, wobei Z_1^t, Z_2^t, Z_3^t und Z_4^t die nativen Plasminogenzwischendomänsequenzen zwischen den Plasminogenkringle-Domänen 1 und 2, 2 und 3, 3 und 4 bzw. 4 und 5 darstellen.

5. Verfahren nach Anspruch 1 zur Herstellung von Plasminogen 1-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon,
Plasminogen 1-544/t-PA 262-527 (arg275 → gln), einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon,
t-PA 1-50/t-PA 88-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon,
t-PA 1-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon, oder
Plasminogen 1-546/u-PA 137-411, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon.

6. Verfahren nach Anspruch 1 zur Herstellung von Plasminogen 1-541/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon.

7. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Wirtszelle eine Ovarzelle des Chinesischen Hamsters ist.

8. Verfahren zur Herstellung eines Hybridplasminogenaktivators nach einem der vorhergehenden Ansprüche, wobei die entfernbare blockierende Gruppe eine 2-Aminobenzoylgruppe ist, die an der 3- oder 4-Position mit einem Halogenatom und außerdem wahlweise mit einer oder mehreren schwach elektronenentziehenden oder elektronenspendenden Gruppe(n) substituiert ist, wobei die Geschwindigkeitskonstante pseudoerster Ordnung der Hydrolyse des Derivats im Bereich von $6,0 \times 10^{-5}$ bis $4,0 \times 10^{-4}$ sec^{-1} liegt bei Messung in einem Puffersystem bestehend aus 0,05 M Natriumphosphat, 0,1 M Natriumchlorid, 0,01 % (Vol./Vol.) Detergens, das Polyoxyethylensorbitanmonooleat mit einem Molekulargewicht von etwa 1300 umfaßt, bei einem pH-Wert von 7,4 bei 37 °C.

9. Verfahren nach Anspruch 8, wobei di entfernbare blockierende Gruppe 4-Fluor-2-aminobenzoyl, 4-Chlor-2-aminobenzoyl oder 4-Brom-2-aminobenzoyl ist.

10. Verfahren nach Anspruch 1 zur Herstellung von 2-Amino-4-chlorbenzoylplasminogen1-544/t-PA 262-527 (zwei Ketten) einschließlich glu_1- und lys_{78}-Varianten und Gemische davon,
2-Amino-4-chlorbenzoylplasminogen 1-541/t-PA 262-527 (zwei Ketten), einschließlich glu_1- und lys_{78}-Varianten und Gemische davon, oder
2-Amino-4-chlorbenzoyl t-PA 1-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527 (zwei Ketten), einschließlich gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon.

11. Verfahren zur Herstellung einer pharmazeutischen Zusammensetzung, die einen wie in einem der vorangegangenen Ansprüche definierten Hybridplasminogenaktivator in Verbindung mit einem pharmazeutisch verträglichen Träger umfaßt, wobei das Verfahren das Vermischen des Aktivators mit dem Träger umfaßt.

12. Verwendung des wie in einem der Ansprüche 1 bis 10 definierten Plasminogenaktivators zur Herstellung eines Medikaments zur Behandlung thrombotischer Krankheiten.

13. Verfahren zur Herstellung eines DNA-Polymers, das eine Nucleotidsequenz umfaßt, die einen wie in einem der Ansprüche 1 bis 6 definierten Hybridplasminogenaktivator codiert, durch die Kondensation geeigneter mono-, di- oder oligomerer Einheiten.

14. Verfahren zur Herstellung eines Derivats eines wie in Anspruch 8 definierten Plasminogenaktivators, wobei das Verfahren die Umsetzung des Plasminogenaktivators mit einem blockierenden Mittel JK umfaßt, wobei J eine lokalisierende Gruppe darstellt, die die Bindung des Mittels an den Katalyseort des Enzyms vermittelt und K eine an der 3- oder 4-Position mit einem Halogenatom und außerdem wahlweise mit einer oder mehreren schwach elektronenentziehenden oder elektronenspendenden Gruppe(n) substituierte 2-Aminobenzoylgruppe darstellt.

15. Verfahren nach Anspruch 14, wobei die Geschwindigkeitskonstante pseudoerster Ordnung im Bereich von $7,0 \times 10^{-5}$ bis $1,5 \times 10^{-4}$ s^{-1} liegt.

16. Verfahren nach Anspruch 14 oder 15, wobei die blockierende Gruppe 4-Fluor-2-aminobenzoyl, 4-Chlor-2-aminobenzoyl oder 4-Brom-2-aminobenzoyl ist.

17. Verfahren nach Anspruch 14 zur Herstellung eines wie in Anspruch 10 definierten Derivats.

Patentansprüche für folgenden Vertragsstaat : GR

1. Hybridplasminogenaktivator, der die fünf Kringle-Domänen von Plasminogen umfaßt, die an die B-Kette von t-PA bzw. u-PA über eine Aminosäuresequenz geknüpft sind, die die t-PA-Spaltstelle zwischen den Resten 275 und 276 und den Cysteinrest 264 von t-PA umfaßt, wobei die Sequenz ausgewählt ist aus:

1. [AAPSTCGLRQYSQPQFR]

2. [AAPSTCGLRQYSQPQFQ]

3. [STCGLRQYSQPQFR]

bzw. die u-PA-Spaltstelle zwischen den Resten 158 und 159 und den Cysteinrest 148 von u-PA umfaßt, wobei die Sequenz ist:
[AAPSFPSSPPEELKFQCGQKTLRPRFK]
(Einbuchstabencode für Aminosäuren)

2. Hybridplasminogenaktivator nach Anspruch 1 mit der Formel:

$(Y-)_m(K^P-Z^l-)_5 B^l$

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wobei B^t die Reste 276-527 von t-PA umfaßt, m 1 ist, die fünf Werte für K^p jeweils ine Kringle-Domäne darst llen und in Folge von Plasminogen abstammen, und Y und j der der 5 Werte für Z^t unabhängig voneinander eine Bindung oder ein verknüpfende Aminosäuresequenz darstellt, die während der Herstellung des Hybridplasminogenaktivators synthetisch eingeführt werden und/oder von nativem Plasminogen und/oder t-PA-Sequenzen abstammen kann, wobei die Sequenz Z_5^t der in Anspruch 1 definierten verknüpfenden Aminosäuresequenz entspricht.

3. Hybridplasminogenaktivator nach Anspruch 2, wobei Y die Plasminogenreste 1 bis 83 bzw. 78 bis 83 darstellt.

4. Hybridplasminogenaktivator nach Anspruch 2 oder 3, wobei Z_1^t, Z_2^t, Z_3^t und Z_4^t die nativen Plasminogenzwischendomänsequenzen zwischen den Plasminogenkringle-Domänen 1 und 2, 2 und 3, 3 und 4 bzw. 4 und 5 darstellen.

5. Plasminogen 1-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon,
Plasminogen 1-544/t-PA 262-527 ($arg\underline{275} \rightarrow gln$), einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon,
t-PA 1-50/t-PA 88-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon,
t-PA 1-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon, oder
Plasminogen 1-546/u-PA 137-411, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon.

6. Plasminogen 1-541/t-PA 262-527, einschließlich Ein- und Zwei-Kettenvarianten, lys_{78}- und glu_1-Varianten und Gemische davon.

7. Hybridplasminogenaktivator nach einem der vorstehenden Ansprüche, exprimiert unter Verwendung von Ovarzellen des Chinesischen Hamsters.

8. Hybridplasminogenaktivator nach einem der vorstehenden Ansprüche, wobei der für die fibrinolytische Aktivität essentielle Katalyseort durch eine entfernbare blockierende Gruppe blockiert ist.

9. Hybridplasminogenaktivator nach Anspruch 8, wobei die entfernbare blockierende Gruppe eine 2-Aminobenzoylgruppe ist, die an der 3- oder 4-Position mit einem Halogenatom und außerdem wahlweise mit einer oder mehreren schwach elektronenentziehenden oder elektronenspendenden Gruppe(n) substituiert ist, wobei die Geschwindigkeitskonstante pseudoerster Ordnung der Hydrolyse des Derivats im Bereich von $6,0 \times 10^{-5}$ bis $4,0 \times 10^{-4}$ sec^{-1} liegt bei Messung in einem Puffersystem bestehend aus 0,05 M Natriumphosphat, 0,1 M Natriumchlorid, 0,01 % (Vol./Vol.) Detergens, das Polyoxyethylensorbitanmonooleat mit einem Molekulargewicht von etwa 1300 umfaßt, bei einem pH-Wert von 7,4 bei 37 °C.

10. Hybridplasminogenaktivator nach Anspruch 9, wobei die entfernbare blockierende Gruppe 4-Fluor-2-aminobenzoyl, 4-Chlor-2-aminobenzoyl oder 4-Brom-2-aminobenzoyl ist.

11. 2-Amino-4-chlorbenzoylplasminogen 1-544/t-PA 262-527 (zwei Ketten) einschließlich glu_1- und lys_{78}-Varianten und Gemische davon,
2-Amino-4-chlorbenzoylplasminogen 1-541/t-PA 262-527 (zwei Ketten), einschließlich glu_1- und lys_{78}-Varianten und Gemische davon, oder
2-Amino-4-chlorbenzoyl-t-PA 1-91/pro-gly-ser/Plasminogen 84-544/t-PA 262-527 (zwei Ketten), einschließlich gly_{-3}-, ser_1- und val_4-Varianten und Gemische davon.

12. Verwendung eines Hybridplasminogenaktivators nach einem der Ansprüche 1 bis 11 zur Herstellung eines Medikaments zur Behandlung von thrombotischen Krankheiten.

13. Verfahren zur Herstellung eines Hybridplasminogenaktivators nach einem der Ansprüche 1 bis 7, wobei das Verfahren die Expression der den Hybridplasminogenaktivator codierenden DNA in einer rekombin-

anten Wirtsz lle und di Gewinnung des Hybridplasminogenaktivatorprodukts umfaßt.

14. DNA-Polymer, das eine Nucleotidsequenz umfaßt, die einen Hybridplasminogenaktivator nach einem der Ansprüche 1 bis 7 codiert.

15. Verfahren zur Herstellung eines Derivats nach Anspruch 8, wobei das Verfahren die Blockierung des für die fibrinolytische Aktivität in dem Hybridplasminogenaktivator nach einem der Ansprüche 1 bis 7 essentiellen Katalyseorts mit einer entfernbaren blockierenden Gruppe umfaßt.

16. Verfahren zur Herstellung eines Derivats nach einem der Ansprüche 9 bis 11, wobei das Verfahren die Umsetzung des Plasminogenaktivators mit einem blockierenden Mittel JK umfaßt, wobei J eine lokalisierende Gruppe darstellt, die die Bindung des Mittels an den Katalyseort des Enzyms vermittelt und K eine an der 3- oder 4-Position mit einem Halogenatom und außerdem wahlweise mit einer oder mehreren schwach elektronenentziehenden oder elektronenspendenden Gruppe(n) substituierte 2-Aminobenzoylgruppe darstellt.

Revendications
Revendications pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, LI, LU, NL, SE

1. Activateur de plasminogène hybride qui comprend les cinq domaines kringles du plasminogène liés à la chaine B du t-PA ou de l'u-PA par l'intermédiaire d'une séquence d'acides aminés qui comprend, respectivement, le site de clivage du t-PA entre les résidus 275 et 276 et le résidu cystéine 264 du t-PA, ladite séquence étant choisie parmi:

1.   [AAPSTCGLRQYSQPQFR]

2.   [AAPSTCGLRQYSQPQFQ]

3.   [STCGLRQYSQPQFR],

ou le site de clivage de l'u-PA entre les résidus 158 et 159 et le résidu cystéine 148 de l'u-PA, ladite séquence étant: [AAPSFPSSPPEELKFQCGQKTLRPRFK] (notation des acides aminés par le code à une lettre)

2. Activateur de plasminogène hybride selon la revendication 1, de formule:

$(Y\text{-})_m(K^P\text{-}Z^t\text{-})_5 B^t$

où B^t comprend les résidus 276-527 du t-PA, m est égal à 1, chacune des 5 valeurs de K^P représente un domaine kringle dérivé du plasminogène en séquence et Y et chacune des 5 valeurs de Z^t représente indépendamment une liaison ou une séquence d'acides aminés de liaison qui peut être introduite par voie de synthèse pendant la préparation de l'activateur de plasminogène hybride et/ou dérivée des séquences du plasminogène natif et/ou du t-PA, la séquence Z_5^t étant la séquence d'acides aminés de liaison définie dans la revendication 1.

3. Activateur de plasminogène hybride selon la revendication 2 dans lequel Y représente respectivement les résidus 1 à 83 ou 78 à 83 du plasminogène respectivement.

4. Activateur de plasminogène hybride selon la revendication 2 ou 3 dans lequel Z_1^t, Z_2^t, Z_3^t et Z_4^t représentent les séquences interdomaines du plasminogène natif entre les domaines kringles 1 et 2, 2 et 3, 3 et 4 et 4 et 5 du plasminogène, respectivement.

5. Plasminogène 1-544/t-PA 262-527, y compris les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges, plasminogène 1-544/t-PA 262-527 (arg_{275} -> gln), y compris les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges,
t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogène 84-544/t-PA 262-527, qui inclut les variants simple et double chaine, les variants gly_{-3}, ser_1 et val_4, et leurs mélanges,
t-PA 1-91/pro-gly-ser/plasminogène 84-544/t-PA 262-527, qui inclut les variants simple et double

chaine, les variants gly$_{-3}$, ser$_1$ et val$_4$, et leurs mélanges ou
plasminogène 1-546/u-PA 137-411, qui inclut les variants simple et doubl chaine, les variants lys$_{78}$ et glu$_1$, t leurs mélanges.

5   6. Plasminogène 1-541/t-PA 262-527, qui inclut les variants simple et double chaine, les variants lys$_{78}$ et glu$_1$, et leurs mélanges.

7. Activateur de plasminogène hybride selon l'une quelconque des revendications précédentes, exprimé en utilisant des cellules d'ovaire de hamster chinois.

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8. Activateur de plasminogène hybride selon l'une quelconque des revendications précédentes, dans lequel le site catalytique essentiel à l'activité fibrinolytique est bloqué par un groupe bloquant qui peut être éliminé.

15   9. Activateur de plasminogène hybride selon la revendication 8 dans lequel le groupe bloquant qui peut être éliminé est un groupe 2-aminobenzoyle substitué en position 3 ou 4 par un atome d'halogène et le cas échéant également substitué par un ou plusieurs groupes faiblement attracteurs d'électrons ou donneurs d'électrons, dans lequel la constante de vitesse d'hydrolyse de pseudo premier ordre du dérivé est comprise entre 6.0×10^{-5} et 4.0×10^{-4} sec^{-1} quand elle est mesurée dans un système
20   tampon constitué par du phosphate de sodium 0.05M, du chlorure de sodium 0.1M, 0.01% v/v de détergent qui comprend du monooléate de polyoxyéthylènesorbitan dont le poids moléculaire est d'environ 1300, à pH 7.4 à 37 ° C.

10. Activateur de plasminogène hybride selon la revendication 9 dans lequel le groupe bloquant qui peut
25   être éliminé est le 4-fluoro-2-aminobenzoyle, le 4-chloro-2-aminobenzoyle ou le 4-bromo-2-aminoben- zoyle.

11. 2-Amino-4-chlorobenzoyl plasminogène 1-544/t-PA 262-527 (double chaine), qui inclut les variants glu$_1$ et lys$_{78}$ et leurs mélanges,
30   2-amino-4-chlorobenzoyl plasminogène 1-541/t-PA 262-527 (double chaine), qui inclut les variants glu$_1$ et lys$_{78}$ et leurs mélanges ou
2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogène 84-544 / t-PA 262-527 (double chaine), qui inclut les variants gly$_{-3}$, ser$_1$ et val$_4$ et leurs mélanges.

35 12. Composition pharmaceutique comprenant un activateur de plasminogène hybride selon l'une quelcon- que des revendications précédentes en combinaison avec un véhicule pharmaceutique acceptable.

13. Activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 11 pour l'utilisation comme substance thérapeutique active.

40

14. Activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 11 pour l'utilisation dans le traitement de maladies thrombotiques.

15. Utilisation d'un activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 11
45   dans la préparation d'un médicament pour le traitement des maladies thrombotiques.

16. Procédé pour la préparation d'un activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 7, lequel procédé comprend l'expression de l'ADN qui code pour ledit activateur de plasminogène hybride dans une cellule hôte recombinante et la récupération du produit activateur de
50   plasminogène hybride.

17. Polymère d'ADN comprenant une séquence nucléotidique qui code pour un activateur de plasminogè- ne hybride selon l'une quelconque des revendications 1 à 7.

55 18. Procédé pour la préparation d'un dérivé selon la revendication 8, lequel procédé comprend le blocage du site catalytique essentiel à l'activité fibrinolytique dans l'activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 7, par un groupe bloquant qui peut être éliminé.

19. Procédé pour la préparation d'un dérivé selon l'une quelconque des revendications 9 à 11, lequl l procédé comprend la réaction de l'activat ur de plasminogène avec un agent bloquant JK dans lequel J est un groupe localisateur qui intervient dans la liaison de l'agent au site catalytique de l'enzyme et K est un groupe 2-aminobenzoyle substitué en position 3 ou 4 par un atome d'halogène et éventuellement également substitué par un ou plusieurs groupes faiblement attracteurs d'électrons ou donneurs d'électrons.

Revendications pour l'Etat contractant suivant : ES

1. Procédé pour la préparation d'un activateur de plasminogène hybride qui comprend les cinq domaines kringles du plasminogène liés à la chaine B du t-PA ou de l'u-PA par l'intermédiaire d'une séquence d'acides aminés qui comprend, respectivement, le site de clivage du t-PA entre les résidus 275 et 276 et le résidu cystéine 264 du t-PA, ladite séquence étant choisie parmi:

$$
\begin{array}{ll}
1. & [\text{AAPSTCGLRQYSQPQFR}] \\
2. & [\text{AAPSTCGLRQYSQPQFQ}] \\
3. & [\text{STCGLRQYSQPQFR}],
\end{array}
$$

ou le site de clivage de l'u-PA entre les résidus 158 et 159 et le résidu cystéine 148 de l'u-PA, ladite séquence étant:
[AAPSFPSSPPEELKFQCGQKTLRPRFK] (notation des acides aminés par le code à une lettre) et dans lequel le site catalytique essentiel à l'activité fibrinolytique est éventuellement bloqué par un groupe bloquant qui peut être éliminé, lequel procédé comprend l'expression de l'ADN qui code pour ledit activateur de plasminogène hybride dans une cellule hôte recombinante et la récupération du produit activateur de plasminogène hybride, éventuellement suivie du blocage du site catalytique essentiel à l'activité fibrinolytique, par un groupe bloquant qui peut être éliminé.

2. Procédé selon la revendication 1 pour la préparation d'un activateur de plasminogène hybride de formule:
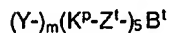
$$(\text{Y-})_m(\text{K}^p\text{-Z}^t\text{-})_5\,\text{B}^t$$

où B^t comprend les résidus 276-527 du t-PA, m est égal à 1, chacune des 5 valeurs de K^p représente un domaine kringle dérivé du plasminogène en séquence et Y et chacune des 5 valeurs de Z^t représente indépendamment une liaison ou une séquence d'acides aminés de liaison qui peut être introduite par voie de synthèse pendant la préparation de l'activateur de plasminogène hybride et/ou dérivée des séquences du plasminogène natif et/ou du t-PA, la séquence Z_5^t étant la séquence d'acides aminés de liaison définie dans la revendication 1.

3. Procédé selon la revendication 2 dans lequel Y représente respectivement les résidus 1 à 83 ou 78 à 83 du plasminogène.

4. Procédé selon la revendication 2 ou 3 dans lequel Z_1^t, Z_2^t, Z_3^t et Z_4^t représentent les séquences inter-domaines du plasminogène natif entre les domaines kringles 1 et 2, 2 et 3, 3 et 4 et 4 et 5 du plasminogène, respectivement.

5. Procédé selon la revendication 1 pour la préparation du plasminogène 1-544/t-PA 262-527, qui inclut les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges, du plasminogène 1-544/t-PA 262-527 (arg 275 -> gln), qui inclut les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges,
du t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogène 84-544/t-PA 262-527, qui inclut les variants simple et double chaine, les variants gly_{-3}, ser_1 et val_4, et leurs mélanges,
du t-PA 1-91/pro-gly-ser/plasminogène 84-544/t-PA 262-527, qui inclut les variants simple et double chaine, les variants gly_{-3}, ser_1 et val_4, et leurs mélanges ou
du plasminogène 1-546/u-PA 137-411, qui inclut les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges.

6. Procédé s lon la revendication 1 pour la préparation du plasminogène 1-541/t-PA 262-527, qui inclut les variants simple et double chaine, les variants lys$_{78}$ et glu$_1$, et leurs mélanges.

7. Procédé selon l'une quelconque des revendications précédentes dans lequel la cellule hôte est une cellule d'ovaire de hamster chinois.

8. Procédé de préparation d'un activateur de plasminogène hybride selon l'une quelconque des revendications précédentes dans lequel le groupe bloquant qui peut être éliminé est un groupe 2-aminobenzoyle substitué en position 3 ou 4 par un atome d'halogène et éventuellement en outre substitué par un ou plusieurs groupes faiblement attracteurs d'électrons ou donneurs d'électrons, dans lequel la constante de vitesse d'hydrolyse de pseudo premier ordre du dérivé est comprise entre 6.0×10^{-5} et 4.0×10^{-4} sec^{-1} quand elle est mesurée dans un système tampon comprenant du phosphate de sodium 0.05M, du chlorure de sodium 0.1M, 0.01% v/v de détergent qui comprend du monooléate de polyoxyethylènesorbitan présentant un poids moléculaire d'environ 1300, à pH 7.4 à 37°C.

9. Procédé selon la revendication 8 dans lequel le groupe bloquant qui peut être éliminé est le 4-fluoro-2-aminobenzoyle, le 4-chloro-2-aminobenzoyle ou le 4-bromo-2-aminobenzoyle.

10. Procédé selon la revendication 1 pour la préparation du 2-amino-4-chlorobenzoyl plasminogène 1-544/t-PA 262-527 (double chaine), qui inclut les variants glu$_1$ et lys$_{78}$ et leurs mélanges, du 2-amino-4-chlorobenzoyl plasminogène 1-541/t-PA 262-527 (double chaine), qui inclut les variants glu$_1$ et lys$_{78}$ et leurs mélanges ou du 2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogène 84-544/t-PA 262-527 (double chaine), qui inclut les variants gly$_{-3}$, ser$_1$ et val$_4$ et leurs mélanges.

11. Procédé pour la préparation d'une composition pharmaceutique qui comprend un activateur de plasminogène hybride tel que défini dans l'une quelconque des revendications précédentes en combinaison avec un véhicule acceptable pharmaceutiquement, lequel procédé comprend le mélange dudit activateur et dudit véhicule.

12. Utilisation d'un activateur de plasminogène hybride tel que défini dans l'une quelconque des revendications 1 à 10 dans la préparation d'un médicament pour le traitement de maladies thrombotiques.

13. Procédé pour la préparation d'un polymère d'ADN comprenant une séquence nucléotidique qui code pour un activateur de plasminogène hybride tel que défini dans l'une quelconque des revendications 1 à 6 par condensation d'unités mono-, di- ou oligomères appropriées.

14. Procédé pour la préparation d'un dérivé d'activateur de plasminogène tel que défini dans la revendication 8, lequel procédé comprend la réaction de l'activateur de plasminogène avec un agent bloquant JK dans lequel J est un groupe localisateur qui intervient dans la liaison de l'agent au site catalytique de l'enzyme et K est un groupe 2-aminobenzoyle substitué en position 3 ou 4 par un atome d'halogène et éventuellement en outre substitué par un ou plusieurs groupes faiblement attracteurs d'électrons ou donneurs d'électrons.

15. Procédé selon la revendication 14, dans lequel la constante de vitesse de pseudo premier ordre est comprise entre 7.0×10^{-5} et 1.5×10^{-4} s^{-1}.

16. Procédé selon la revendication 14 ou 15 dans lequel le groupe bloquant est le 4-fluoro-2-aminobenzoyle, le 4-chloro-2-aminobenzoyle ou le 4-bromo-2-aminobenzoyle.

17. Procédé selon la revendication 14 pour la préparation d'un dérivé tel que défini dans la revendication 10.

Revendications pour l'Etat contractant suivant : GR

1. Activateur de plasminogène hybride qui comprend les cinq domaines kringles du plasminogène liés à la chaine B du t-PA ou de l'u-PA par l'intermédiaire d'une séquence d'acides aminés qui comprend, respectivement, le site de clivage du t-PA entre les résidus 275 et 276 et le résidu cystéine 264 du t-PA, ladite séquence étant choisie parmi:

1.   [AAPSTCGLRQYSQPQFR]

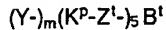2.   [AAPSTCGLRQYSQPQFQ]

3.   [STCGLRQYSQPQFR],

ou le site de clivage de l'u-PA entre les résidus 158 et 159 et le résidu cystéine 148 de l'u-PA, ladite séquence étant:

[AAPSFPSSPPEELKFQCGQKTLRPRFK] (notation des acides aminés par le code à une lettre)

2. Activateur de plasminogène hybride selon la revendication 1, de formule:

$(Y-)_m(K^P-Z^t-)_5 B^t$

où B^t comprend les résidus 276-527 du t-PA, m est égal à 1, chacune des 5 valeurs de K^P représente un domaine kringle dérivé du plasminogène en séquence et Y et chacune des 5 valeurs de Z^t représente indépendamment une liaison ou une séquence d'acides aminés de liaison qui peut être introduite par voie de synthèse pendant la préparation de l'activateur de plasminogène hybride et/ou dérivée des séquences du plasminogène natif et/ou du t-PA, la séquence Z_5^t étant la séquence d'acides aminés de liaison définie dans la revendication 1.

3. Activateur de plasminogène hybride selon la revendication 2 dans lequel Y représente respectivement les résidus 1 à 83 ou 78 à 83 du plasminogène respectivement.

4. Activateur de plasminogène hybride selon la revendication 2 ou 3 dans lequel Z_1^t, Z_2^t, Z_3^t et Z_4^t représentent les séquences interdomaines du plasminogène natif entre les domaines kringles 1 et 2, 2 et 3, 3 et 4 et 4 et 5 du plasminogène, respectivement.

5. Plasminogène 1-544/t-PA 262-527 qui inclut les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges,
plasminogène 1-544/t-PA 262-527 (arg 275 -> gln) incluant les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges,
t-PA 1-50/t-PA 88-91/pro-gly-ser/plasminogène 84-544/t-PA 262-527 incluant les variants simple et double chaine, les variants gly_{-3}, ser_1 et val_4, et leurs mélanges,
t-PA 1-91/pro-gly-ser/plasminogène 84-544/t-PA 262-527 incluant les variants simple et double chaine, les variants gly_{-3}, ser_1 et val_4, et leurs mélanges ou
plasminogène 1-546/u-PA 137-411 incluant les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges.

6. Plasminogène 1-541/t-PA 262-527 incluant les variants simple et double chaine, les variants lys_{78} et glu_1, et leurs mélanges.

7. Activateur de plasminogène hybride selon l'une quelconque des revendications précédentes exprimé en utilisant des cellules d'ovaire de hamster chinois.

8. Activateur de plasminogène hybride selon l'une quelconque des revendications précédentes, dans lequel le site catalytique essentiel à l'activité fibrinolytique est bloqué par un groupe bloquant qui peut être éliminé.

9. Activateur de plasminogène hybride selon la revendication 8, dans lequel le groupe bloquant qui peut être éliminé est un groupe 2-aminobenzoyle substitué en position 3 ou 4 par un atome d'halogène et éventuellement en outre substitué par un ou plusieurs groupes faiblement attracteurs d'électrons ou donneurs d'électrons, dans lequel la constante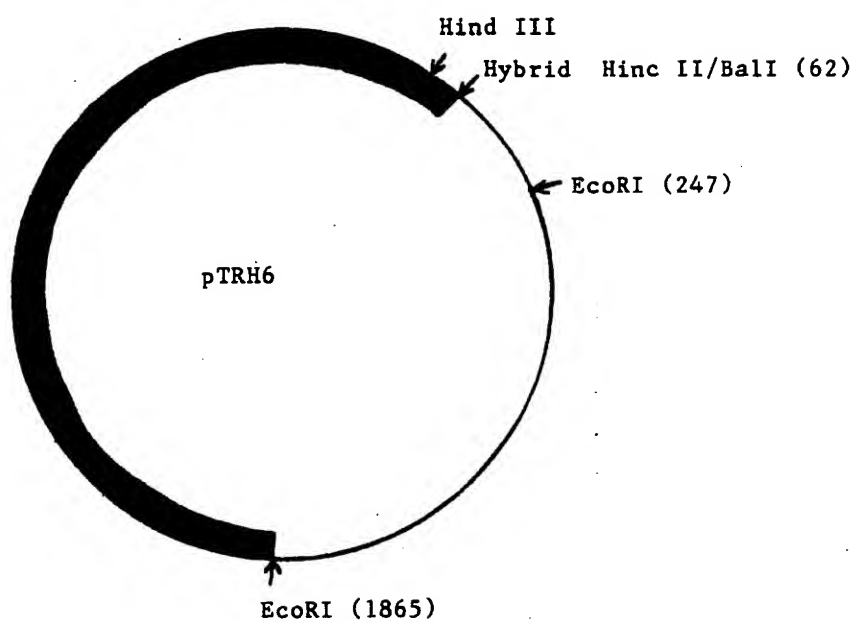 de vitesse d'hydrolyse de pseudo premier ordre du dérivé est comprise entre 6.0×10^{-5} et 4.0×10^{-4} sec^{-1} quand elle est mesurée dans un système tampon comprenant du phosphate de sodium 0.05M, du chlorure de sodium 0.1M, 0.01% v/v de détergent qui comprend du monooléate de polyoxyethylènesorbitan dont le poids moléculaire est d'environ 1300, à pH 7.4 à 37·C.

10. Activateur de plasminogène hybrid selon la revendication 9 dans l quel l group bloquant qui peut être éliminé est le 4-fluoro-2-aminob nzoyle, l 4-chloro-2-aminobenzoyle ou le 4-bromo-2-aminobenzoyle.

11. 2-Amino-4-chlorobenzoyl plasminogène 1-544/t-PA 262-527 (double chaine), incluant les variants glu_1 et lys_{78} et leurs mélanges, 2-amino-4-chlorobenzoyl plasminogène 1-541/t-PA 262-527 (double chaine), incluant les variants glu_1 et lys_{78} et leurs mélanges ou 2-amino-4-chlorobenzoyl t-PA 1-91/pro-gly-ser/plasminogène 84-544 / t-PA 262-527 (double chaine), incluant les variants gly_{-3}, ser_1 et val_4 et leurs mélanges.

12. Utilisation d'un activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 11 dans la préparation d'un médicament pour le traitement des maladies thrombotiques.

13. Procédé pour la préparation d'un activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 7, lequel procédé comprend l'expression d'ADN qui code pour ledit activateur de plasminogène hybride dans une cellule hôte recombinante et la récupération du produit activateur de plasminogène hybride.

14. Polymère d'ADN comprenant une séquence nucléotidique qui code pour un activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 7.

15. Procédé pour la préparation d'un dérivé selon la revendication 8, lequel procédé comprend le blocage du site catalytique essentiel à l'activité fibrinolytique dans l'activateur de plasminogène hybride selon l'une quelconque des revendications 1 à 7 par un groupe bloquant qui peut être éliminé.

16. Procédé pour la préparation d'un dérivé selon l'une quelconque des revendications 9 à 11, lequel procédé comprend la réaction de l'activateur de plasminogène avec un agent bloquant JK dans lequel J est un groupe localisateur qui intervient dans la liaison de l'agent au site catalytique de l'enzyme et K est un groupe 2-aminobenzoyle substitué en position 3 ou 4 par un atome d'halogène et éventuellement en outre substitué par un ou plusieurs groupes faiblement attracteurs d'électrons ou donneurs d'électrons.
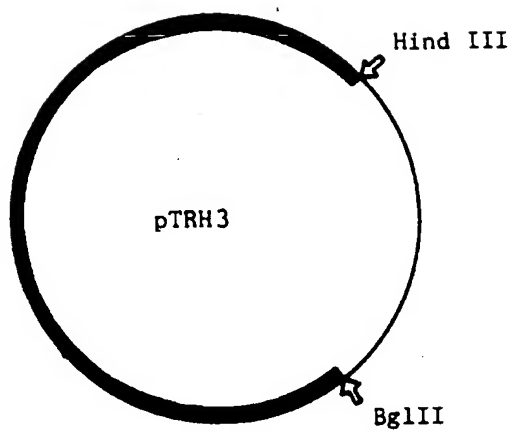
Fig. 1a

Hind III

Hybrid Hinc II/BalI (62)
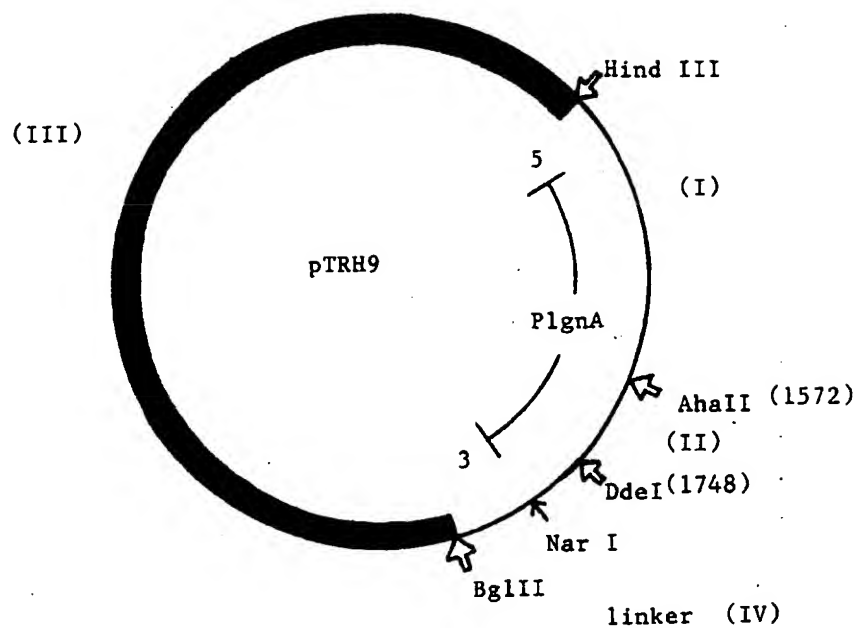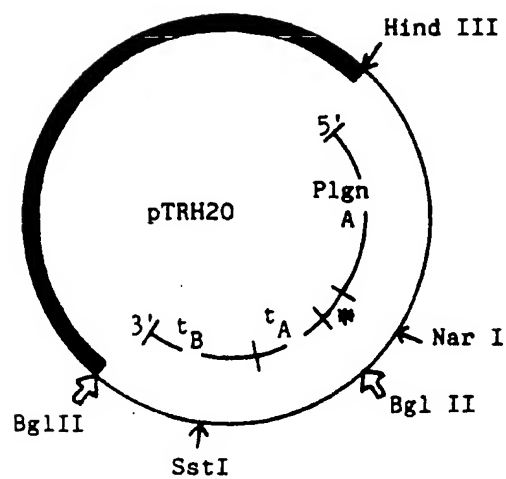
EcoRI (247)

pTRH6

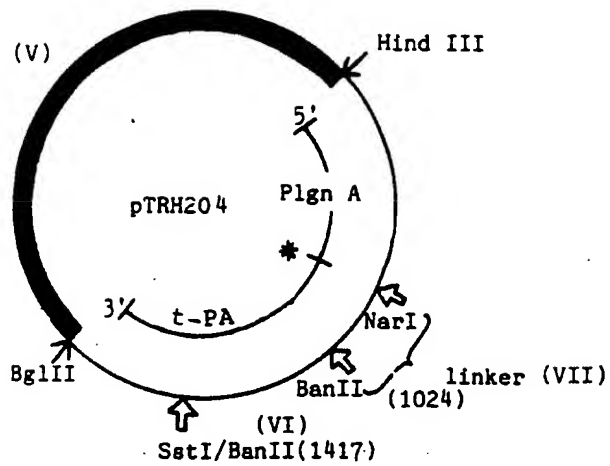EcoRI (1865)

Fig. 1b

Hind III

pTRH3

BglII

Fig. 2

Fig. 3

Fig.4

Fig. 5

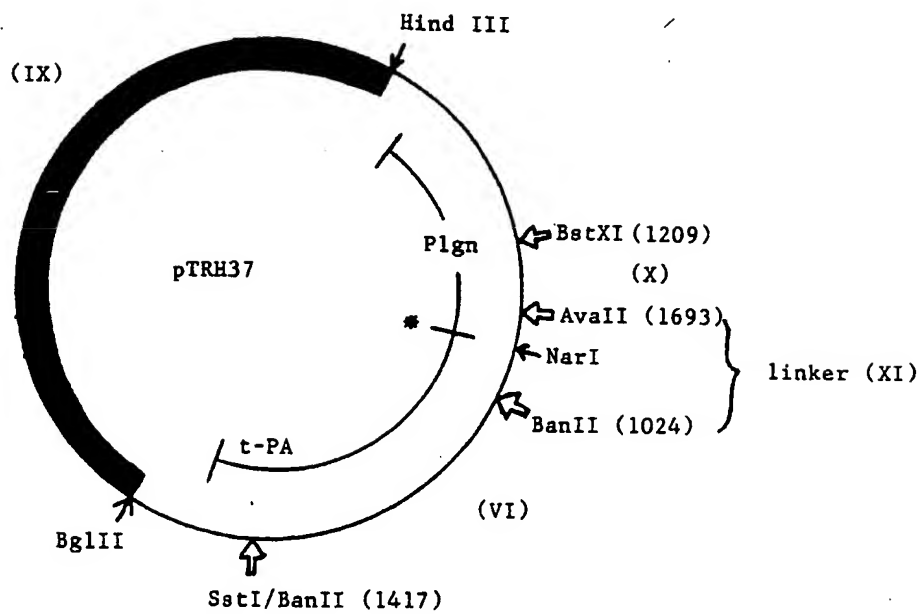Fig. 6

Fig. 7

Fig. 8

pTRH01

Hind III

Fragment II

Fragment I

BamH1

Linker

KpnI

Fig. 9

pTRH02

HindIII

Fragment1

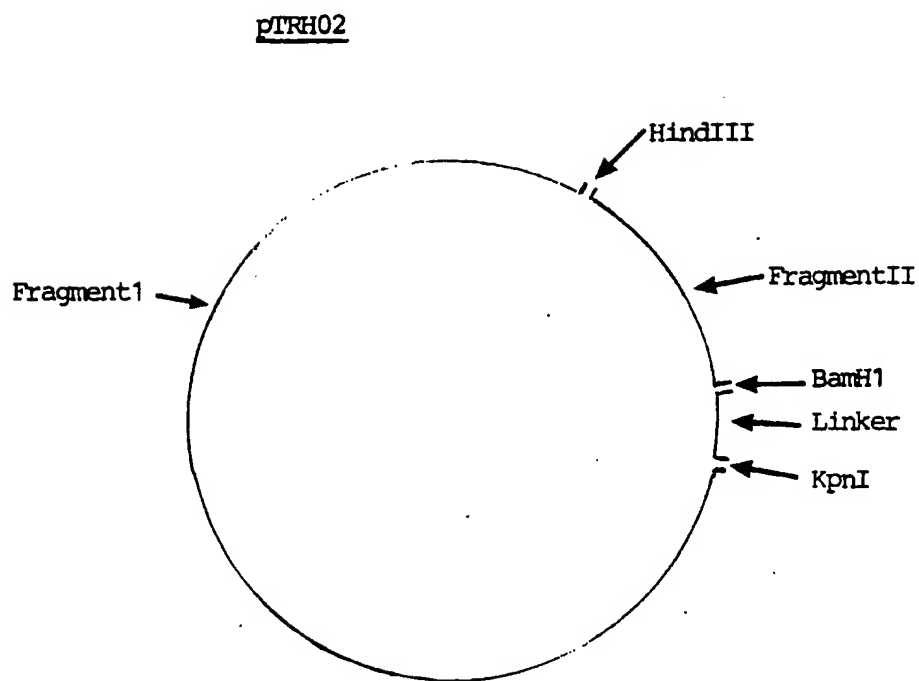FragmentII

BamH1

Linker
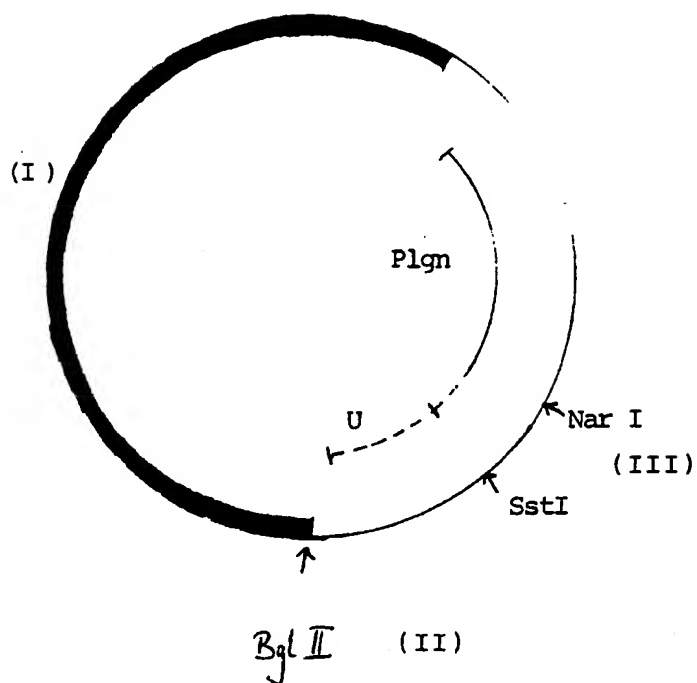
KpnI

Fig.10

pTRH25
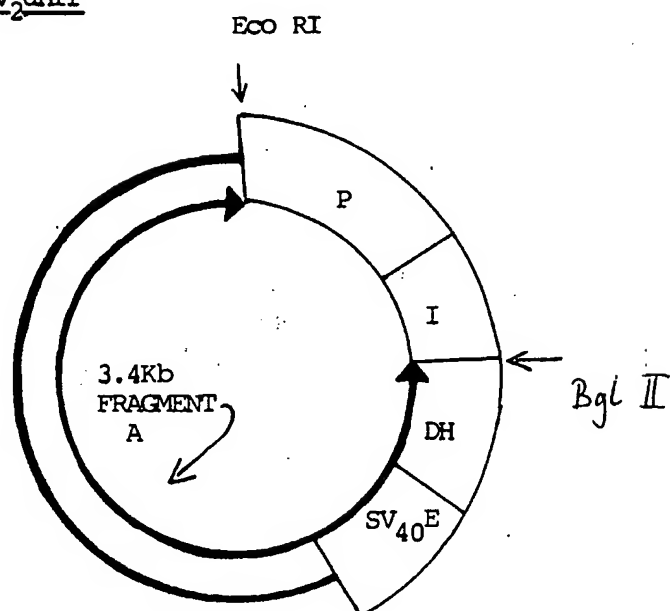

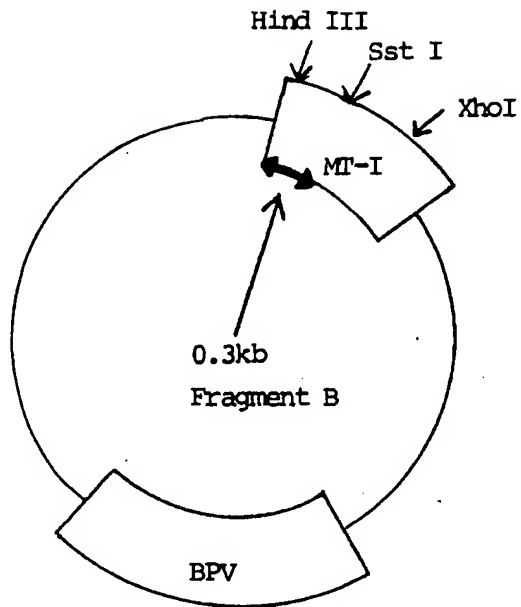Bgl II (II)
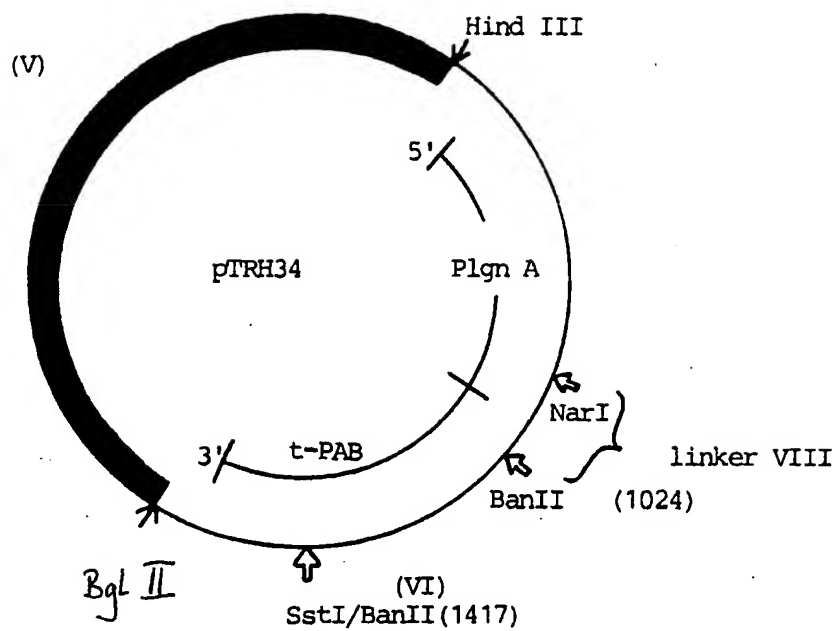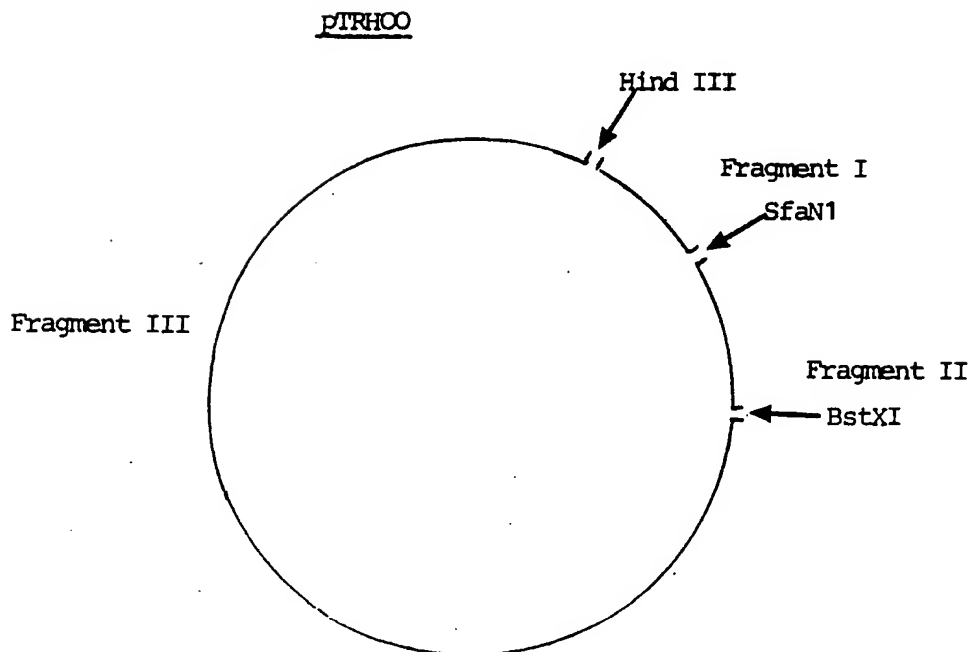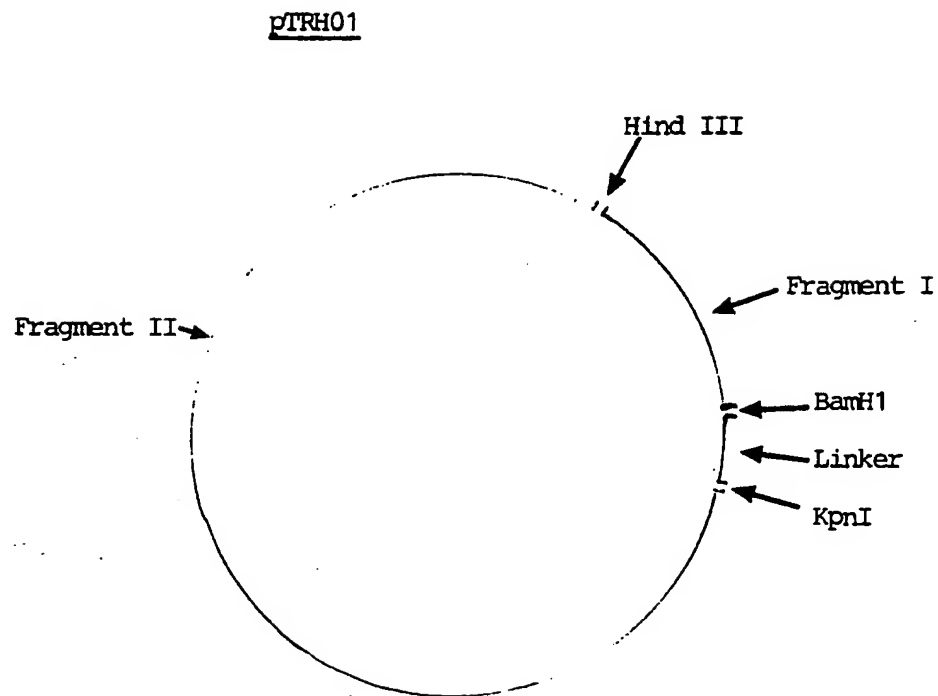
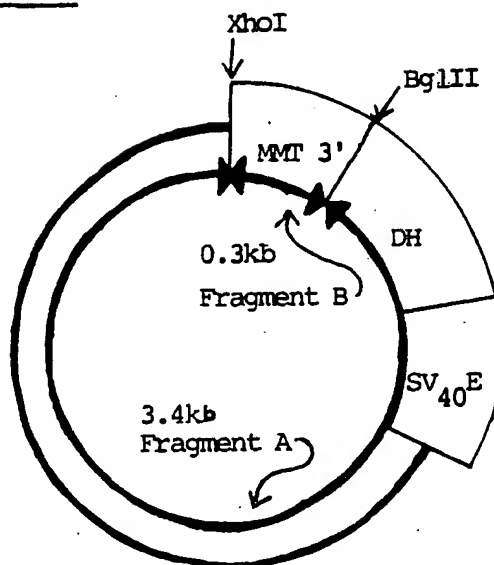Fig. 11

Plasmid PSV$_2$dhfr

Fig. 12

Plasmid BPV-MT-XhoI
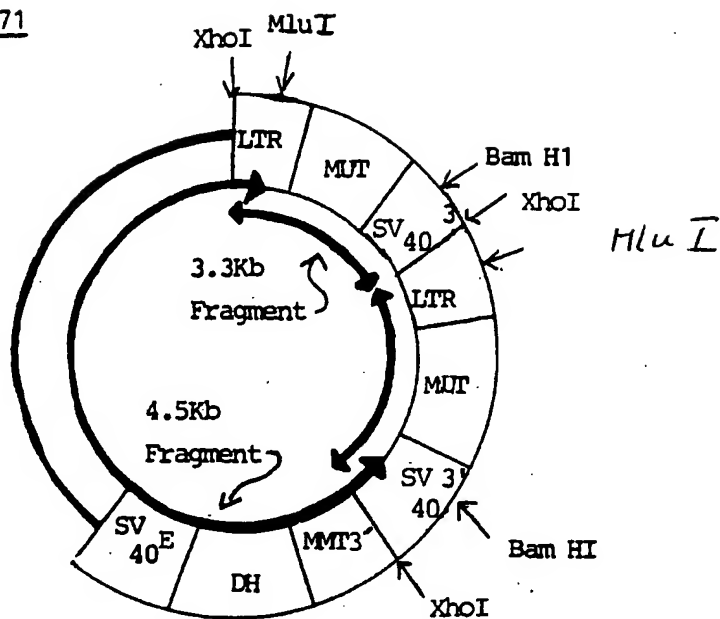

Fig. 13

Plasmid pTRH69

Fig. 14

Plasmid pTRH71

Fig. 15

Plasmid pTHRH11